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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 February 2004 (12.02.2004)

PCT

(10) International Publication Number
WO 2004/013070 A2

(51) International Patent Classification[7]: C07B 61/00

(21) International Application Number:
PCT/DK2003/000516

(22) International Filing Date: 30 July 2003 (30.07.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2002 01171 1 August 2002 (01.08.2002) DK
60/399,692 1 August 2002 (01.08.2002) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 2004/013070 A2

(54) Title: MULTI-STEP SYNTHESIS OF TEMPLATED MOLECULES

(57) Abstract: Disclosed is a method for the manufacture of a library of complexes. The complexes comprise templated molecules attached to the template which directed the synthesis thereof. The templated molecules are produced in a step-by-step fashion which provides for a high local concentration of reactive groups involved in the formation of connections between the individual components of the template molecule.

Multi-step synthesis of templated molecules.

**Technical Field**

The present invention relates to a method for the manufacture of a library of complexes. Each complex in the library comprises a templated molecule attached to the template which directed the synthesis thereof. The library of the invention is useful in the quest for new biological active compounds, such as drugs.

**Background art**

The generation of molecules carrying new properties remains a challenging task. Recently, a number of procedures have been suggested that should allow a more efficient generation and screening of a larger number of molecules. The approaches taken involve the encoding and/or templating of molecules other than natural biopolymers such as peptide, RNA and DNA. These approaches allow the researcher to generate and screen a huge number of molecules in a short time. This should lead to better molecules carrying the desired properties.

The central dogma of biology describes the one-way flow of information from DNA to RNA to protein. Recently, methods such as phage display, peptides-on-plasmids, ribosome display and mRNA-protein fusion have been developed, allowing the transfer of information from the level of protein/peptide to RNA or DNA. This has enabled the use of molecular evolution to be applied on huge numbers of peptides that are exposed to an enrichment process, where after the enriched pool of molecules (enriched for a particular feature, such as binding to receptor protein) are amplified, by exploiting information flow from the peptide to DNA and then amplifying the DNA.

More recently, approaches have been developed that allow the encoding of polypeptides and other biochemical polymers. An example of this approach is

disclosed in US 5,723,598, which pertains to the identification of a biochemical polymer that participates in a preselected binding interaction with a target to form a binding reaction complex. The prior art method encompasses the generation of a library of bifunctional molecules. One part of the bifunctional molecule is the biochemical polymer and the other part is an identifier oligonucleotide comprising a sequence of nucleotides which encodes and identifies the biochemical polymer. Following the generation of the library of the bifunctional molecules, a partitioning with respect to affinity towards the target is conducted and the identifier oligonucleotide part of the bifunctional molecule is amplified by means of PCR. Eventually, the PCR amplicons are sequenced and decoded for identification of the biochemical polymer. This approach suffers from the draw-back that it is necessary with a laborius decoding step following each round of selection. Thus the flow of information from the identifier sequence to the biochemical polymer is restrained.

Halpin and Harbury have in WO 00/23458 suggested an improvement to the approach stipulated immediately above, wherein the molecules formed are not only identified but also directed by the nucleic acid tag. The approach is based on the traditional split-and-combine strategy for synthesis of combinatorial libraries comprising two or more synthetic steps. Plurality nucleic acid templates are used, each having at one end a chemical reactive site and dispersed throughout the stand a plurality of codons regions, each of said codon regions in turn specifying different codons. Separately, each of the strands, identified by a first codon region, is reacted at the chemical reaction sites with specific selected reagents. Subsequently, all the strands are pooled and subjected to a second partitioning based on a second codon region. The split-and-combine method is conducted an appropriate number of times to produce a library of typically between $10^3$ and $10^6$ different compounds. The split-and-combine method is cumbersome and generates only a relatively small library.

The present invention aims at obtaining a library of compounds which are not only encoded by a suitable tag attached to each compound, but also directed. The directed synthesis of the compounds of the library allows for renewed synthesis of templated molecules following a selection round. Furthermore, the present invention increases the probability of connection of molecular entities eventually appearing in the templated molecule due to a higher local concentration of the reactive groups involved in the formation of the connection.

**Disclosure of the invention**

The present invention relates to a method for the manufacture of a library of complexes comprising templated molecules, said method comprises the steps of

a) providing a plurality of different templates comprising a number of coding regions and a reactive group, wherein each coding region of a specific template specifies a unique codon,

b) providing a plurality of different building blocks, each building block comprising an anti-codon, a functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon of each building block complements a unique codon of a template, and the functional entity comprises at least one reactive group,

c) contacting the plurality of different templates with a subset of the plurality of different building blocks, said subset having anti-codons which complement the unique codons of a specific coding region, said contacting being performed under conditions which allow specific hybridisation of the anti-codons to the unique codons of the templates,

d) reacting the reactive group of the template and the reactive group of the building block to obtaining a chemical connection,

e) contacting under conditions allowing specific hybridisation, the plurality of different templates harbouring the nascent templated molecules with a further subset of the plurality of building blocks, said subset having anti-codons complementary to the unique codons of a coding

region in the vicinity of the coding region harbouring the nascent templated molecules,

f) allowing the functional entities of the subset of further building blocks to form a chemical connection to the nascent templated molecules,

g) optionally, cleaving one or more of the linkers, provided that at least one linker remains to connect the nascent templated molecule with the template which directed the synthesis thereof,

h) optionally repeating steps e) through g),

i) obtaining a templated molecule attached via the linker one or more building blocks to the template which directed the synthesis thereof.

The present invention allow for a multi-step templated synthesis of a library of molecules without the need for a laborious and time consuming split step of the traditional split and-mix-method. The method furthermore provides for the possibility of bringing the functional entities of building blocks in close proximity, thus allowing facilitated connection of functional entities in the vicinity of each other. The bringing in close proximity of building blocks provide for an increased local concentration of the active chemical groups, thus increasing the probability that two reactive groups will be so close that a reaction actually will occur.

The various templates of the present invention are in general constructed to follow a general scheme. According to the scheme, a number of coding regions are provided on the template. In turn, each of the coding regions specifies one or more unique codons. Thus, a specific template comprises a given number of unique codons. The plurality of templates can, taken as a whole, be characterized as a library comprising the total amount of the different combinations of unique codons possible, or any subset thereof. The coding regions are suitable positioned in a linear sequence, such that the individual coding regions are positioned immediately next to each other. In some embodiments, it may be of advantage to use a branched template to ensure

proximity of reactive groups, the introduction of catalysts in the vicinity of the reactive groups or the introduction of as third reactant.

Besides the coding regions, the templates used in the present invention include a reactive group. The reactive group comprised by the template may be covalently or non-covalently attached to the template. Covalent attachment may be preferred when the templated molecule is to be effectively attached to the template, because a covalent bonding will allow affinity selection using more harsh conditions. The covalent attachment of the reactive group may be done at a terminal region of the template or at a central region thereof. In an aspect of the invention, the reactive group is non-covalently attached to the template using a complementing element hybridised to the template. More particularly, it is preferred that the reactive group of the template is part of a building block hybridised to the template.

The coding regions may be spaced with a suitable spacer region. The spacer region may be an indentifier for the coding region of may be a region not carrying any information but serving to bring the functional entities into the desired proximity or to provide the template with a desired physical characteristic like a stiff connection of coding regions, or alternative, a flexible connection between two coding regions.

The template may comprise flanking regions. One of the flanking regions can in an aspect of the invention serve to immobilize the template to a surface of a solid support. In another aspect of the invention the flanking region can encompasses a signal group, such a flourophor or a radio active group, to allow a direct detection of the presence of the template.

The plurality of templates used in the present invention may in one embodiment be represented by the general formula:

F-(Coding region 1)-($S^1$)-(Coding region 2)-($S^2$)...-(Coding region n)-($S^n$)-F'

Wherein

Each of Coding region 1 through Coding region n independently specifies m unique codons,

F and F' are optional flanking regions,

$S^1$ to $S^n$ are optional spacing groups,

n is an integer of at least 2, and

m is an integer of at least 1.

The unique codons of the templates are preferably composed of a sequence of nucleic acid monomers, such as nucleotides. Each codon is unique in the sense that within the same coding region no other codons have an identical sequence and length of nucleic acid monomers. Preferably, a unique codon does not have a corresponding sequence anywhere in the plurality of templates. To avoid hybridisation between individual templates it is also desirable to design each of the unique codons such that the complementary sequence thereof does not exist on any other templates.

The number of coding regions may be selected in accordance with *inter alia* the number of the desired final templated compounds, the building blocks available and the envisaged structure of the templated compound. According to the invention the number of coding regions is preferably at least 3 to achieve the desired diversity. The upper limit for the number of coding regions has not yet been elucidated; however it is believed that a number exceeding 100 may give practical problems. Generally, it is preferred to use templates having between 3 and 50 coding regions, more preferably between 3 and 30 and still more preferred between 4 and 15.

Within each of the coding regions the number of unique codons may be selected according to the need for diversity. The number of unique codons in each of the coding regions may be similar or different. The number of unique codons can be as low as one. This may be the choice when a so-called scaf-

fold is involved in the evolving templated molecule. The upper limit for the number of unique codons may be chosen quit high as long as specific hybridisation of oligonucleotides of the anti-codons to their complements on the templates occurs. An example of an upper limit may be 10,000, but may be chosen below this limit or above according to the need.

The building blocks essential for the present invention, are generally composed of three elements, *viz.* an anti-codon, a functional entity, and a linker which connects the anti-codon and the functional entity. The anti-codon is a sequence of nucleic acid monomers complementary to the sequence of a unique codon on at least one of the plurality of templates. In one aspect of the invention, building blocks are provided which possesses anti-codons complementing all the unique codons of the variety of templates. In another aspect, some of the building blocks carrying anti-codons for selected unique codons are not present. The latter situation may occur when termination of the synthesis is desired at different stages or when the absences of certain functional entities on the templated molecule are desirable. Furthermore, anti-codons not associated with any functional entity may be present for steric reasons or to avoid unintended hybridisation events.

The design of the anti-codons and the complementing unique codons may be aimed at obtaining essentially the same annealing temperature for all or some of the codon:anti-codon hybrids to ensure that all the anti-codons have been annealed to the template before the functional entities are connected to each other through a chemical reaction. In an aspect of the invention, the annealing temperature of the codon:anti-codon hybrids within the same coding region is designed to have different annealing temperature. The separate reaction may be accompliced by initially raising the temperature above the annealing temperature for all the hybrids and slowly decreasing the temperature until the first set of anti-codons anneal to its templates. Following the connection of the functional entity to another functional entity or a nascent templated molecule, the temperature is decreased sufficient for allowing an-

other building blocks to anneal to a coding region in the vicinity. The above design provides for the possibility that functional groups of different templates but within the same coding region are subjected to different reaction conditions.

A further design involves annealing temperatures different for each of the coding regions but similar within a specific coding region. Upon proper design it is possible step-wise to anneal the individual building blocks by gradually decreasing the temperature from above the annealing temperature for the total of the codon:anti-codon hybrids to a temperature at or below a temperature where all the codon:anti-codon hybrids have been formed within the same coding region. Subsequently or simultaneously with the temperature regime selected, a connection between the functional entities and the other functional entities or nascent templated molecules is performed. At each step of the step-wise decrease of the temperature, a new building block is annealed to the template and a subsequent connection is performed. This design allows for the simultaneous addition of all the building blocks to the plurality of templates and, thus, omitting the step-wise addition of building blocks.

It is within the capability of the skilled person in the art to construct the desired design. When a specific annealing temperature is desired it is a standard procedure to suggest appropriate compositions of nucleic acid monomers and the length thereof. The construction of an appropriate design may be assisted by software, such as Vector NTI Suite or the public database at the internet address http://www.nwfsc.noaa.gov/protocols/oligoTMcalc.html.

The functional entity of the building block serves the function of being a precursor for the structural entity eventually incorporated into the templated molecule.

The functional entity is designed to be capable of being connected to a functional entity of another building block or a nascent templated molecule. The connection is aided by one or more reactive groups of the functional entity. The number of reactive groups which appear on the functional entity is suitably one to ten. A building block featuring only one reactive group is used *i.a.* in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. Two or more reactive groups intended for the formation of connections, are typically present on scaffolds. A scaffold is a core structure, which forms the basis for the creation of multiple variants. The variant forms of the scaffold is typically formed through reaction of reactive groups of the scaffold with reactive groups of other building blocks, optionally mediated by fill-in groups or catalysts, under the creation of a connection between the entities. The functional entities to be connected to the scaffold may contain one, two or several reactive groups able to form connections.

Some of the linkers are durable during the entire synthesis to ensure the connection between the scaffolded molecule and the template. It is essential for the invention that at least one linker is maintained in order to ensure the coupling of the templated molecule to the template which directed the synthesis thereof. In a final state of the production of the templated molecule the at least one linker emanating from a building block may be substituted by another entity securing the adherence of the template molecule to the template. The durable linkers are preferably cleavable at a final stage to separate the templated molecule from the template or a complementary template. Therefore, the durable linkers may be referred to as selectively cleavable linkers. Preferably, some of the linkers of the building blocks are also cleavable during the synthesis of the templated molecule, *i.e.* the functional entity may be released from the building block to allow the synthesis of the templated molecule.

The linker may be attached to the anti-codon at a central area thereof or at one of the ends. In one aspect of the invention, the anti-codon and the linker is a contiguous oligonucleotide, *i.e.* a part of the nucleotide complements a sequence of the template and another part is non-complementing avoiding the hybridisation of the oligonucleotide part to the template. This design of the building blocks is a convenient way of design as no separate reaction step is required for the attachment of the linker to the anti-codon. In another aspect of the invention the linker is attached to a central part of the anti-codon to allow for the ligation of neighbouring anti-codons using suitable enzymes to produce a complementary template.

The linker can be attached to the functional entity according to the functionalities desired. In one aspect, the linker is attached to the functional entity through a reactive group capable of forming a connection to another functional entity or a nascent templated molecule. Examples of suitable reactive groups are imine groups (-NH-) and disulfide groups (-S-S-). The bonding of the functional entity to the linker can be cleaved simultaneously with the reaction of the inter-spacing reactive group or the cleavage can be performed in a separate step. In the following, linkers connected to a functional entity through a reactive group which is cleaved simultaneously with the formation of the connection, are referred to as translocating linkers. Translocating linkers allow for the production of templated polymers, which are connected to the template that directed the synthesis thereof via the terminal building block, when a reactive group on a functional entity in the vicinity reacts to form a connection. The separate formation of the connection between a functional entity and another functional entity or evolving templated molecule and the cleavage of the linker is an advantage because more than one connection may be formed prior to the cleavage.

A subset of the building blocks is contacted with the plurality of templates in the initial phase of the production of the library. The subset of the total amount of building blocks is selected to have anti-codons which complement

unique codons of a specific coding region on the template. It may be of advantage to have the building blocks in the vicinity of the reactive group of the template. In the event, the reactive group of the template is a part of a building block, it is preferred that the building blocks to be linked together is attached in the vicinity of each other to ensure a sufficient proximity of the functional entities. Preferably, the subset comprises building blocks having anti-codons which form hybrids with unique codons in two neighbouring coding regions. The subset may be provided by adding the building blocks separately, or alternatively, by adding all the building blocks or a major portion thereof and then direct the annealing of the individual building blocks by proper design of the codon:anti-codon hybrids, as depicted above.

The conditions which allow specific hybridisation of the unique codons and the anti-codons are influenced by a number of factors including temperature, salt concentration, type of puffer, and acidity. It is within the capabilities of the person skilled in the art to select appropriate conditions to ensure that the contacting between the templates and the building blocks are performed at hybridisation conditions.

When two building blocks in the initial stage of the present method are hybridised to a template the functional entities of each of the building blocks are allowed to form a chemical connection. The connection between two functional entities usually occurs by a reaction between reactive groups present on each functional entity. It may, however, be desirable to make the bridging between two reactive groups on separate functional entities through a suitable fill-in group. The latter situation may occur, for example, when two similar reactive groups, such as a two amine groups, are not able to react with each other directly. The two amine groups may, however, be connected to each other through a di-carboxylic acid, such as oxalic acid, to create amide bondings.

Following the successful connection of two entities for the formation of the nascent templated compound, one of the linkers connected thereto may be cleaved. However, this step is optional. It is possible to continue with the incorporation of further building blocks without this cleaving step. In one aspect of the invention, the connection of the functional entities and the cleavage of one of the linkers occur simultaneously. This aspect of the invention is of particular relevance when the reactive group able to react with another reactive group of a functional entity in the vicinity thereof, is the bridging group to the linker.

The propagation part of the method is initiated by contacting the plurality of different templates harbouring the nascent templated compound with a further subset of the plurality of building blocks, said subset having anti-codons complementary to the unique codons of a coding region in the vicinity of the coding region(s) harbouring the nascent templated molecules. To increase the proximity, it can be suitable to hybridise the further building blocks to a coding region neighbouring the building block(s) harbouring the nascent templated molecule. The subset of building blocks may be provided by adding building blocks separately which hybridise to a specific coding region. Alternatively, different stringency conditions combined with appropriate design of the individual codon:anti-codon hybrids can be selected to allow the predominate annealing of a subset of building blocks to selected codon regions. The alternative way of forming a subset has the advantage that all or at least a major part of the building blocks can be added to the reaction vessel. An example of directing the annealing is to design the first two coding regions such that they have a relatively high annealing temperature, whereas the subsequent building blocks to be annealed have a gradually decreasing annealing temperature.

When the further building block is hybridised to a template also harbouring the evolving templated molecule, the functional entity of the further building block is allowed to form a chemical connection to the nascent templated

molecule. The formation of the chemical connection normally proceeds by reaction between reactive groups present on the functional entity and the nascent templated molecule, respectively. It may, however, be desirable to make the bridging between two reactive groups through a suitable spacer group. The latter situation may for example occur when two similar reactive groups, such as a two amine groups, are not able to react with each other directly. The two amine groups may, however, be connected to each other through a di-carboxylic acid, such as oxalic acid, to create amide bondings.

After the connection between the nascent templated molecule and the further functional entity one or more of the linkers may optionally be cleaved, however ensuring that at least one linker is durable. The propagation part of the method may be repeated a desired number of times to evolve the templated molecule. Each repetition of the propagation is initiated by contacting the templated with a new subset of further building blocks.

After the propagation stage follows the termination stage. Depending on the degree of cleavage during the propagation, the attachment of the templated molecule or the complementary template, is different. At one extreme none of the linkers are cleaved during the synthesis, which is when the optional cleavage of linkers of step g) is not performed. This may lead to a templated molecule attached to a number of linker similar to the number of building blocks involved in the synthesis. At the other extreme, the complexes obtained in step i) comprise templated molecules attached to the template which templated the syntheses thereof via the linker of a single building block. The number of linkers may be anywhere between these two extremes in the templated molecule. In some applications it is of advantage to have more than one linker, *e.g.* when the conformation of the templated molecule can be stabilized or even altered. In general, it is sufficient for the templated molecule to be attached to the template through a single linker to allow the subsequent enrichment process to proceed efficiently.

The attachment via a building block involves the use of hydrogen bondings between the codon and the anti-codon to ensure the coupling of the template and the templated molecule. As is well-known within the art, hydrogen bondings are weak bondings that may easily be disrupted. Therefore, in an aspect of the invention, the building block finally harbouring the templated molecule, may be attached to the template through a codon:anti-codon hybrid having a higher annealing temperature than the other codon:anti-codon hybrids of the template. Alternatively, and in some applications preferably, the templated molecule is connected with the template which directed the syntheses thereof via a covalent link. The covalent link may be in addition to the hydrogen bondings or the covalent link may be a substitution. The presence of a covalent link allows for a more harsh chemical treatment of the complex. In one aspect of the invention, the covalent link is selectively cleavable to provide for a separation of the templated molecule from the complementary template.

The method according to the present invention may involve the further step of cleaving all but one linker after the formation of the templated molecule. This further step implies that some or all of the linkers in step g) are not cleaved during the synthesis of the templated molecule.

The method according to the invention may, as a further step, involve the transfer of the templated molecule to an anchorage point on the template, or a sequence complementing the template, to establish an effective chemical connection between the template and the templated molecule. An effective coupling of the templated molecule to the template or a sequence complementary to the template can be desirable to allow for denaturing enrichment conditions or denaturing post-templating modification of the manufactured molecule. The anchorage may involve the presence of a reactive group on the templated molecule and a reaction partner on the template, whereby the reaction between these reactive groups will establish a covalent link. Alternatively, the anchorage point may be present on a complementary sequence hybridised to the template. In a preferred embodiment the complementing

sequence has a higher annealing temperature than one or more of the building blocks, notably the terminal building block, to enable usage of a higher stringency during enrichment and , optionally, clearance of used building blocks.

The anti-codons can, after the cleavage of the linker, remain hybridised to the unique codons or can be detached from the template. When it is chosen to detach the anti-codons, they are preferably cleared from the solution to avoid any re-hybridisation or interference with nucleic acids intended to participate in an hybridisation event. In an aspect of the invention, the anti-codons following the cleavage of the linker attached thereto, remain hybridised to the unique codons because the anti-codons attached to the templates can be ligated together to create a complementary template. The ligation of the anti-codons may be performed after all or the majority of building blocks have been incorporated or, in the alternative, the ligation can be performed after the incorporation of each new building block. Furthermore, in some occasions, it may be of advantage to ligate the anti-codons together prior to the cleavage of the linkers. The ligation of the all the anti-codons provide a direct covalent link between the complementing template and the templated molecule. The covalent link is preferably designed to allow the separation of the templated molecule from the complementing template. The separation of the templated molecule will in general during an enrichment process appear as one of the later steps. Therefore, it is of importance in most applications that the covalent link is selectively cleavable, i.e. cleavable under certain chemical conditions not used in the prior steps of enrichment. In one aspect of the invention the templated molecule is released by the use of enzymes. As an example, restriction nucleases may be used by the incorporation of a restriction site close to the templated molecule. Another example is to use a phosphodiesterase to perform a total or partly digest of the template or complementing template.

The use of ligation also have another advantage, because it is possible to use anti-codons having a shorter sequence of nucleotides. An example of a typically anti-codon will have 15 to 25 nucleotides in sequence to obtain an appropriate annealing temperature of around 40 to 70°C. By ligating building blocks in the vicinity of each other using a ligase or a chemical crosslink, it becomes possible to incorporate very small anti-codons (e.g. 4-10 nucleotides) with high specificity and efficiency. The ligation of a small anti-codon to another anti-codon or a complementing template increases the total annealing temperature. A result of using smaller anti-codons is that, the local concentration of functional entities is increased, and therefore, the efficiency of the reaction between the reactive groups becomes more efficient.

Another way to increase the proximity further is to provide building blocks intended to interact with each other, with a reversible interacting molecule pair. The molecule pair allows a building block to form a reversible coupling to another building block in the vicinity thereof through the interaction of the two parts of the pair situated on each building block. Preferably, the molecule pair is also termed a dimerisation domain and is located in the functional entity or is a portion of the linker that is close to the functional entity. The dimerisation domains of two building blocks intended to react with each other are designed to have an affinity to each other. Examples of dimerisation domains include leucine-rich areas, coiled-coil structures, antibody-antigen pairs, complementing sequences of nucleotides, ect. The affinity of the dimerisation of two building blocks are preferably lower than the affinity between the codon and the anti-codon to allow for shifting dimerisation partners though performing temperature cycles. When the dimerisation domains of two building blocks intended to interact are sequences of nucleotides, the length of the sequences may be chosen to obtain an annealing temperature below room temperature but preferably above 5°C, *e.g.* between 10°C and 20°C. When the dimerisation domain includes two complementing oligonucleotides, the domain is also referred to as a "zipper box".

A suitable temperature scheme for the propagation step of the present invention is to add a building block to the template at a temperature above the annealing temperature for said building block. Then the temperature is lowered below the annealing temperature to allow the new building block as well as the building block harbouring the nascent templated molecule to find and bind to their respective parts of the template. Excess building blocks and debris are then preferably washed away. Then the temperature is decreased below the annealing temperature of the dimerisation domain and consequently the probability that the functional entities are connected is increased. Potentially, the conditions in the reaction vessel, other than the temperature, may be changed to provide for the connection. Following the connection between the functional entities/templated molecules, the temperature is raised and the temperature scheme is repeated.

Another method of increasing the proximity further is to apply a "rigid" linker attached to the anti-codon with molecular hinges. A result of using a rigid linker is that a smaller three dimensional space is sampled by the functional entity. Thereby the probability is increased that two functional entities in the vicinity of each other and attached to a rigid linker will be close enough to each other for a reaction to proceed. A rigid linker may be prepared by various methods available to the skilled person in the art. An example is to use a double stranded oligonucleotide. In a preferred embodiment, the anti-codon and the linker is performed of a contiguous oligonucleotide, wherein the anti-codon domain of the oligonucleotide is able to hybridise to a unique codon of a template and a stiffer domain is able to hybridise to a further oligonucleotide complementary thereto. The hinges may be provided by any group allowing essentially unhindered rotation about at least one bonding. A hinge may be provided in the above preferred embodiment by separation of the anti-codon domain and the stiffer domain with at least one nucleotide. In other words, a hinge may be provided by a single stranded region positioned between the double stranded unique codon:anti-codon hybrid and the double stranded rigid linker.

In one aspect of the invention, the template is covalently connected to the complementing template. The connection may be performed by covalently bonding the two hybridised strings to each other. In the alternatively, the template may at one end be designed with a hair pin loop to enable the ligation of the template end to an anti-codon. According to this aspect, the templated molecule will be linked to a double stranded template. The double stranded template may be an advantage because it is more stable allowing more versatile chemical reactions.

In one aspect of the invention a library of complexes is obtainable from the above methods.

The library may be used for a variety of applications, including the search for compounds for use in therapeutic or diagnostic methods and plant protection compounds, like pesticides, fungicides ect. The library may comprise any number of complexes according to the invention. At one extreme, the library consists of only two complexes. At the other extreme, the library can consist of up to $10^{18}$ complexes. Usually, the number of complexes is to be selected between these to extremes.

One method to identify the most active compounds which can be used in *e.g.* therapeutic applications is to subject the library to an enrichment treatment. According to one aspect of the invention an enrichment of a library of complexes comprising templated molecules with respect to a predetermined activity, comprises the steps of:

i) establishing a first library of complexes comprising templated molecules, said library being obtainable according to any of the methods of the invention,

ii) exposing the library to conditions enriching the library with complexes having the predetermined activity,

iii) amplifying the complexes of the enriched library,

iv) optionally, repeating step ii) to iii), and

v) obtaining an enriched library having a higher ratio of complexes comprising templated molecules with the predetermined activity.

The amplification is normally preferred, though not always necessary. Especially, when several cycles of enrichments are conducted it is of advantage to make an amplification to obtain sufficient complexes. In a preferred aspect of the invention, the amplification of the complexes of the enriched library comprises the steps of contacting the library of complexes with amplification means, amplifying the templates or the complementing templates, and conducting the method according to the invention using the amplification product as templates. The amplification means can be any of the nucleic acid amplification means suitable for the amplification of the template, such as PCR. Preferably, the amplification of the complex comprises a $10^1$ to $10^{15}$-fold amplification.

To allow for multiple enrichment cycles the steps ii) and iii) are repeated at least 2, 3, 5 times, such as at least 10 times, such as at least 15 times. The complexes may be identified after the completion of each cycle or may be only be identified after the last cycle. There is no explicit need for intermediate identifications as the amplification can be performed without knowing the sequence of the template or a sequence complementing the template, if the template or the complement thereof is provided with suitable primer regions. The identification after the enrichment process involves the determination of the sequence of the template and/or the structural determination of the templated molecule and/or the entire complex having the predetermined activity.

Preferably, the conditions enriching the library comprise contacting a binding partner to the templated molecules of interest. The binding partner may be in solution or may be directly or indirectly immobilised on a support. The enrichment is in general performed using an affinity or activity assay. In one

aspect of the invention, the enrichment is conducted by screening for complexes having an affinity for – or an effect on – a target molecule or a target entity. In another aspect the enrichment is conducted by selection for catalytic activity. Alternatively, the conditions enriching the library involves any one or more of electrophoretic separation, gelfiltration, immunoprecipitation, isoelectric focusing, centrifugation, and immobilization.

The enrichment process can involve cells. Thus, in one embodiment, the conditions enriching the library comprises providing cells capable of internalising the templated molecule, or performing an interaction with the templated molecule having the desired predetermined activity.

When the library of complexes have been enriched to a small pool comprising complexes displaying a predetermined activity, it is desirable to obtain each of the complexes separately. Thus, the invention also entails to a method for the manufacture of a complex of a templated molecule attached to the template which directed the synthesis thereof, said method comprises the steps of

a) providing a template comprising a number of coding regions and a reactive group, wherein each coding region specifies a unique codon,
b) providing a plurality of different building blocks, each building block comprising an anti-codon, a functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon of each building block complements a unique codon of the template, and the functional entity comprises at least one reactive group,
c) contacting the template with a building block having an anti-codon which complements the unique codon of a specific coding region, said contacting being performed under conditions which allow specific hybridisation of the anti-codon to the unique codon of the templates,
d) reacting the reactive group of the template and the reactive group of the building block to obtaining a chemical connection,

e) contacting under conditions allowing specific hybridisation, the template harbouring the nascent templated molecule with a further building block having an anti-codon complementary to the unique codon of a coding region in the vicinity of the coding region harbouring the nascent templated molecule,

f) allowing the functional entity of the further building block to form a chemical connection to the nascent templated molecule,

g) optionally, cleaving one or more of the linkers, provided that at least one linker remains to connect the nascent templated molecule with the template which directed the synthesis thereof,

h) optionally repeating steps e) through g),

i) obtaining a templated molecule attached via the linker of one or more building blocks to the template which directed the synthesis thereof.

The templated molecule can be obtained from the complex by cleaving the linker(s) of the one or more building blocks to release the templated molecule from the template.

**Detailed disclosure of the invention**

Codon

The codons occurring in nature consist of a sequence of three nucleic acid monomers. The length of the codon sequence may be of the same order in accordance with the present invention but is preferably longer to obtain a suitable annealing temperature. Preferably the sequence is selected to produce an annealing temperature above normal room temperature. Herein the terms annealing temperature and melting temperature may be used interchangeably said temperature being defined as the maximum of the first derivative of the absorbance *vs.* temperature curve. The different codons need no to be of the same lengths, that is to comprise the same number of nucleic acid monomers. However, a typically number of nucleic acid monomers in the codon sequence is normally above 6 but below 25.

Each nucleic acid monomer is normally composed of three parts, namely a nucleobase moiety, a sugar moiety and a internucleoside linker.

The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. It should be clear to the person skilled in the art that various nucleobases which previously have been considered "nonnaturally occurring" have subsequently been found in nature. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-$N^6$-methyladenine, 7-deazaxanthine, 7-deazaguanine, $N^4$,$N^4$-ethanocytosin, $N^6$,$N^6$-ethano-2,6-diamino-purine, 5-methylcytosine, 5-($C^3$-$C^6$)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover every and all of these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, 5-methylcytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

The sugar moiety is suitably a pentose but may be the appropriate part of an PNA. Suitable examples of possible pentoses include ribose, 2'-deoxyribose, 2'-O-methyl-ribose, 2'-flour-ribose, and 2'-4'-O-methylene-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity. An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer. The internucleoside linkage may be the natural occurring phospodiester linkage or a derivative thereof. Examples of such derivatives include phosphorothioate, methylphosphonate, phosphoramidate, phosphotriester, and phosphodithioate. Furthermore, the internucleoside

linker can be any of a number of non-phosphorous-containing linkers known in the art.

Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine.

Each codon is complemented by an anti-codon. The anticodon has the ability specifically to engage with the codon which it complements. The affinity between the codon and the complementing anti-codon is affected through hydrogen bondings following the well-known Watson-Crick base pairing system. Thus, the anti-codon may be composed of the same kind of nucleic acid monomers as the codon itself.

Linkers

Linkers connecting the anti-codon and functional entity of building blocks may be selected from a variety of possibilities. Linkers may include one or more reactive groups in order to obtain a selectively cleavable linker, a cleavable linker, and a translocating linkers. Suitable linkers may be selected from but are not limited to, the group comprising: carbohydrides and substituted carbohydrides; vinyl, polyvinyl and substituted polyvinyl; acetylene, polyacetylene; aryl/hetaryl, polyaryl/hetaryl and substituted polyaryl/polyhetaryl; ethers, polyethers such as e.g. polyethylenglycol and substituted polyethers; amines, polyamines and substituted polyamines; double stranded, single stranded or partially double stranded natural and unnatural polynucleotides and substituted double stranded, single stranded or partially double stranded natural and unnatural polynucleotides; and polyamides and natural and un-

natural polypeptides and substituted polyamides and natural and unnatural polypeptides.

Functional groups

The functional entity may comprise one or more functional groups, i.e. groups which eventually form part of the templated molecule. The templated molecule may comprise one or more of the following functional groups either alone or in combination:

1. Hydroxyls
2. Primary, secondary, tertiary amines
3. Carboxylic acids
4. Phosphates, phosphonates
5. Sulfonates, sulfonamides
6. Amides
7. Carbamates
8. Carbonates
9. Ureas
10. Alkanes, Alkenes, Alkynes
11. Anhydrides
12. Ketones
13. Aldehydes
14. Nitatrates, nitrites
15. Imines
16. Phenyl and other aromatic groups
17. Pyridines, pyrimidines, purines, indole, imidazole, and heterocyclic bases
18. Heterocycles
19. polycycles
20. Flavins
21. Halides

22. Metals
23. Chelates
24. Mechanism based inhibitors
25. Small molecule catalysts
26. Dextrins, saccharides
27. Fluorescein, Rhodamine and other fluorophores
28. Polyketides, peptides, various polymers
29. Enzymes and ribozymes and other biological catalysts
30. Functional groups for post-polymerization/post activation coupling of functional groups
31. Drugs, e.g., taxol moiety, acyclovir moiety, "natural products"
32. Supramolecular structures, e.g. nanoclusters
33. Lipids
34. Oligonucleotides, oligonucleotide analogs (e.g., PNA, LNA, morpholinos)
35. Hydrogen

Reactive groups

Reactive groups relates among other things to groups which form part of the functional entity and are capable of participating in a reaction that form a connection between two functional entities, either directly or via a suitable bridging molecular entity. Examples of reactive groups are listed below:

1. N-carboxyanhydrides (NCA)
2. N-thiocarboxyanhydrides (NTA)
3. Amines
4. Carboxylic acids
5. Ketones
6. Aldehydes
7. Hydroxyls
8. Thiols
9. Esters
10. Thioesters

11. conjugated system of double bonds
12. Alkyl halides
13. Hydrazines
14. N-hydroxysuccinimide esters
15. Epoxides
16. Haloacetyls
17. UDP-activated saccharides
18. Sulfides
19. Cyanates
20. Carbonylimidazole
21. Thiazinanones
22. Phosphines
23. Hydroxylamines
24. Sulfonates
25. Activated nucleotides
26. Vinylchloride
27. Alkenes, quinones

Templated molecules

According to the present invention, virtually any molecule may be templated using the general method disclosed herein. Examples of compounds which it is anticipated can be synthesised includes, but are not limited to, the compounds listed below:

alpha-, beta-, gamma-, and omega-peptides Mono-, di- and tri-substituted peptides; L- and D-form peptides; Cyclohexane- and cyclopentane-backbone modified beta-peptides; Vinylogous polypeptides; glycopolypeptides; polyamides; vinylogous sulfonamide peptide; polysulfonamide; conjugated peptide (i.e., having prosthetic groups); polyesters; polysaccharides; polycarbamates; polycarbonates; polyureas; poly-peptidylphosphonates; azatides; peptoids (oligo N-substituted glycines); polyethers; ethoxyformacetal oligomers; poly-

thioethers; polyethylene glycols (PEG); polyethylenes; polydisulfides; polyarylene sulfides; polynucleotides; PNAs; LNAs; morpholinos; oligo pyrrolinone; polyoximes; polyimines; polyethyleneimine; polyacetates; polystyrenes; polyacetylene; polyvinyl; lipids; phospholipids; glycolipids; polycycles (aliphatic); polycycles (aromatic); polyheterocycles; proteoglycan; polysiloxanes; polyisocyanides; polyisocyanates; polymethacrylates; monofunctional, Difunctional, Trifunctional and Oligofunctional open-chain hydrocarbons; monofunctional, difunctional, trifunctional and oligofunctional nonaromatic carbocycles; monocyclic, bicyclic, tricyclic and polycyclic hydrocarbons; bridged polycyclic hydrocarbons; monofunctional, difunctional, trifunctional, and oligofunctional nonaromatic heterocycles; monocyclic, bicyclic, tricyclic, and polycyclic heterocycles, bridged polycyclic heterocycles; monofunctional, difunctional, trifunctional and oligofunctional aromatic carbocycles; monocyclic, bicyclic, tricyclic, and polycyclic aromatic carbocycles; monofunctional, difunctional, trifunctional and oligofunctional aromatic heterocycles; monocyclic, bicyclic, tricyclic and polycyclic heterocycles; chelates; fullerenes; steroids; cyclosporin analogs; as well as any combination of the above molecular moieties.

Use of Library

Selection or screening, commonly referred to as enrichment, of the library of complexes comprising templated molecules with respect to desired activities (for example binding to particular target, catalytic activity, or a particular effect in an activity assay) may be performed according to any standard protocol. For example, affinity selections may be performed according to the principles used for phage displayed, polysome-displayed or mRNA-protein fusion displayed peptides. Selection for catalytic activity may be performed by affinity selections on transition-state analogue affinity columns (Baca et al. , Proc. Natl. Acad. Sci USA. 1997; 94(19):10063-8), or by function-based selection schemes (Pedersen et al., Proc. Natl. Acad. Sci. USA. 1998, 95(18):10523-8). Screening for a desired characteristic may be performed according to standard microtiter plate-based assays, or by FACS-sorting assays.

Generally, affinity selections involve the immobilisation of a target or a binding partner on a solid support, such as a column. Subsequently, the complex manufactured according to the invention is added to the column under conditions allowing a part of the complexes to bind to the target. The complexes not bound to the target is eluted out of the column and discharged. The part of the complexes attached to the target may be amplified using the template or complementing template associated with the templated molecule.

The choice of amplification method depends on the choice of codons and anti-codons. Natural oligonucleotides can be amplified by any state of the art method. These methods include, but is not limited to the polymerase chain reaction (PCR); as wells as e.g. nucleic acid sequence-based amplification (e.g. Compton, Nature 350, 91-92 (1991)), amplified anti-sense RNA (e.g. van Gelder et al., PNAS 85: 77652-77656 (1988)); self-sustained sequence replication system (e.g. Gnatelli et al., PNAS 87: 1874-1878 (1990)); polymerase independent amplification as described in e.g. Schmidt et al., NAR 25: 4797-4802 (1997), as well as in vivo amplification of plasmids carrying cloned DNA fragments. Ligase-mediated amplification methods may also be used, e.g., LCR (Ligase Chain Reaction).

For non-natural nucleotides the choices of efficient amplification procedures are fewer. As non-natural nucleotides per definition can be incorporated by certain enzymes including polymerases, it will be possible to perform manual polymerase chain reaction by adding the polymerase during each extension cycle.

For oligonucleotides containing nucleotide analogs, fewer methods for amplification exist. One may use non-enzyme mediated amplification schemes (Schmidt et al., NAR 25: 4797-4802 (1997)). For backbone-modified oligonucleotide analogs such as PNA and LNA, this amplification method may be used. Before or during amplification the templates or

complementing templates may be mutagenized or recombined in order to create a larger diversity for the next round of selection or screening.

Following the amplification of the template part or complementing template part of the complex, the method according to the invention is conducted using the amplification product as the templates. The result is a reduced or enriched library of complexes of a template attached to a template molecule.

The selection and amplification steps may be repeated if considered necessary to further enrich the library. When the selection and amplification steps are repeated, the binding step involving the target and the complexes, is preferably performed under more strict conditions ensuring that only a part of the complexes adhere to the target.

The enrichment cycles may be performed 2 to 15 times or even more with enrichment in each cycle of 10 to 1000 times. In one approach, the starting library amounts to $10^{14}$ complexes. After seven cycles of enrichments with a 100 fold concentration in each cycle, the complex with the highest affinity to the target should, theoretically, be obtained. However, it is more likely that the final cycles deliver a small pool of interesting complexes, which have to be examined by other means.

After the final round of selection, it is often desirable to sequence individual templates, in order to determine the composition of individual templated molecules. If the template contains natural nucleotides, it is a standard routine to optionally PCR amplify the isolated templates (if the template is an RNA molecule, it is necessary to use reverse transcriptase to produce cDNA prior to the PCR-amplification), and then clone the DNA fragments into for example plasmids, transform these and then sequence individual plasmid-clones containing one or multiple tandem DNA sequences. In this case, it is practical to design a restriction site in both of the flanking sequences to the central random or partly random sequence of the template (i.e., in the primer

binding sites). This will allow easy cloning of the isolated nucleotides. Sequencing can be done by the standard dideoxy chain termination method, or by more classical means such as Maxam-Gilbert sequencing.

If the template contains non-natural nucleotides, it may not be feasible to clone individual sequences by transfer through a microbial host. However, using bead populations where each bead carries one oligonucleotide sequence, it is possible to clone in vitro, where after all the nucleotides attached to a specific bead may be optionally amplified and then sequenced (Brenner et al., 2000, Proc. Natl. Acad. Sci. USA 97, 1665-1670). Alternatively, one may dilute the population of isolates adequately, and then aliquot into microtiter plates so that the wells on average contain for example 0.1 templates. By amplifying the single templates by for example PCR, it will now be possible to sequence using standard methods. Of course, this requires that the non-natural nucleotides are substrates for the thermostable polymerase used in the PCR.

If alternative methods are used that require shorter oligonucleotides it may be desirable to design the starting template so as to contain restriction sites on either side of the encoding/templating region of the template. Thereby, after the final selection round, the templates can be restricted, to obtain a short oligonucleotide encoding the templated polymer, and then these short oligonucleotides can be applied to various analytical procedures.

It is also possible to sequence the isolates by the use of a DNA array of oligonucleotides with random but predetermined sequences.

It may also be desirable to sequence the population of isolates as a pool, for example if the sequences are expected to be in register, for example because the initial library consisted of a degenerate sequence based on a polymer sequence with a known (relatively high) desired activity. Therefore, it is then expected that all the isolates have sequences similar to the initial

sequence of the templates before selection. Thus, the population of isolates can be sequenced as a whole, to obtain a consensus sequence for the population as a whole.

*Selection of template-displaying molecules that will bind to known targets*

The present invention is also directed to approaches that allow selection of small molecules capable of binding to different targets. The template-displaying molecule technology contains a built-in function for direct selection and amplification. The binding of the selected molecule should be selective in that they only coordinate to a specific target and thereby prevent or induce a specific biological effect. Ultimately, these binding molecules should be possible to use e.g. as therapeutic agents, or as diagnostic agents.

Template-displaying molecule libraries can easily be combined with screenings, selections, or assays to assess the effect of binding of a molecule ligand on the function of the target. In a more specific embodiment, the template-displaying method provides a rapid means for isolating and identifying molecule ligands which bind to supra-molecular, macro-supra-molecular, macro-molecular and low-molecular structures (*e.g.* nucleic acids and proteins, including enzymes, receptors, antibodies, and glycoproteins); signal molecules (*e.g.* cAMP, inositol triphosphate, peptides, prostaglandins); and surfaces (*e.g.* metal, plastic, composite, glass, ceramics, rubber, skin, tissue).

Specifically, selection or partitioning in this context means any process whereby the template-displaying molecule complex bound to a target molecule, the complex-target pair, can be separated from template-displaying molecules not bound to the target molecule. Selection can be accomplished by various methods known in the art.

The selection strategy can be carried out so it allows selection against almost any target. Importantly, no steps in this selection strategy need any detailed

structural information of the target or the molecules in the libraries. The entire process is driven by the binding affinity involved in the specific recognition/coordination of the molecules in the library to a given target. However, in some applications, if needed, functionality can also be included analogous to selection for catalytic activity using phage display (Soumillion et al. (1994) J. Mol. Biol. 237: 415-22; Pedersen et al. (1998) PNAS. 18: 10523-10528). Example of various selection procedures are described below.

This built-in template-displaying molecule selection process is well suited for optimizations, where the selection steps are made in series starting with the selection of binding molecules and ends with the optimized binding molecule. The single procedures in each step are possible to automate using various robotic systems. This is because there is a sequential flow of events and where each event can be performed separately. In a most preferable setting, a suitable template-displaying molecule library and the target molecule are supplied to a fully automatic system which finally generates the optimized binding molecule. Even more preferably, this process should run without any need of external work outside the robotic system during the entire procedure.

The libraries of template-displayed molecules will contain molecules that could potentially coordinate to any known or unknown target. The region of binding on a target could be into a catalytic site of an enzyme, a binding pocket on a receptor (*e.g.* GPCR), a protein surface area involved in protein-protein interaction (especially a hot-spot region), and a specific site on DNA (*e.g.* the major groove). The template-displaying molecule technology will primarily identify molecules that coordinate to the target molecule. The natural function of the target could either be stimulated (agonized) or reduced (antagonized) or be unaffected by the binding of the template-displaying molecules. This will be dependent on the precise binding mode and the particular binding-site the template-displaying molecules occupy on the target. However, it is known that functional sites (*e.g.* protein-protein interaction or catalytic sites) on different proteins are more prone to bind molecules that

other more neutral surface areas on a protein. In addition, these functional sites normally contain a smaller region that seems to be primarily responsible for the binding energy, the so called hot-spot regions (Wells, et al. (1993) Recent Prog. Hormone Res. 48; 253-262). This phenomenon will increase the possibility to directly select for small molecules that will affect the biological function of a certain target.

The template-displaying molecule technology of the invention will permit selection procedures analogous to other display methods such as phage display (Smith (1985) Science 228: 1315-1317). Phage display selection has been used successfully on peptides (Wells & Lowman. (1992) Curr. Op. Struct. Biol. 2, 597-604) proteins (Marks et al. (1992) J. Biol. Chem. 267: 16007-16010) and antibodies (Winter et al. (1994) Annu. Rev. Immunol. 12: 433-455). Similar selection procedures are also exploited for other types of display systems such as ribosome display (Mattheakis et al. (1994) Proc. Natl. Acad. Sci. 91: 9022-9026) and mRNA display (Roberts, et al. (1997) Proc. Natl. Acad. Sci. 94: 12297-302). However, the template-displaying molecule technology of the invention, will for the first time allow direct selection of target-specific small non-peptide molecules independently of the translation process on the ribosome complex. The necessary steps included in this invention are the amplification of the templates and incorporation and reaction of the monomer building blocks. The amplification and incorporation and the incorporation and reaction are either done in the same step or in a sequential process.

The linkage between the templated molecule (displayed molecule) and DNA replication unit (coding template) allows a rapid identification of binding molecules using various selection strategies. This invention allows a broad strategy in identifying binding molecules against any known target. In addition, this technology will also allow discovery of novel unknown targets by isolating binding molecules against unknown antigens (epitopes) and use these bind-

ing molecules for identification and validation (see section "*Target identification and validation*").

As will be understood, selection of binding molecules from the template-displaying molecule libraries can be performed in any format to identify optimal binding molecules. A typical selection procedure against a purified target will include the following major steps: Generation of a template-displaying molecule library: Immobilization of the target molecule using a suitable immobilization approach; Adding the library to allow binding of the template-displayed molecules; Removing of the non-binding template-displayed molecules; Elution of the template-displayed molecules bound to the immobilized target; Amplification of enriched template-displaying molecules for identification by sequencing or to input for the next round of selection. The general steps are schematically shown in Figure 27.

In a preferred embodiment, a standard selection protocol using a template-displaying molecule library is to use the bio-panning method. In this technique, the target (*e.g.* protein or peptide conjugate) is immobilized onto a solid support and the template-displayed molecules that potentially coordinate to the target are the ones that are selected and enriched. However, the selection procedure requires that the bound template-displayed molecules can be separated from the unbound ones, i.e. those in solution. There are many ways in which this might be accomplished as known to ordinary skilled in the art.

The first step in the affinity enrichment cycle (one round as described in Figure 27) is when the template-displayed molecules showing low affinity for an immobilized target are washed away, leaving the strongly binding template-displayed molecules attached to the target. The enriched population, remaining bound to the target after the stringent washing, is then eluted with, e.g. acid, chaotropic salts, heat, competitive elution with the known ligand or proteolytic release of the target/template molecules. The eluted template-

displayed molecules are suitable for PCR, leading to many orders of amplification, i.e. every single template-displayed molecule enriched in the first selection round participates in the further rounds of selection at a greatly increased copy number. After typically three to ten rounds of enrichment a population of molecules is obtained which is greatly enriched for the template-displayed molecules which bind most strongly to the target. This is followed quantitatively by assaying the proportion of template-displaying molecules which remain bound to the immobilized target. The variant template sequences are then individually sequenced.

Immobilisation of the target (peptide, protein, DNA or other antigen) on beads might be useful where there is doubt that the target will adsorb to the tube (e.g. unfolded targets eluted from SDS-PAGE gels). The derivatised beads can then be used to select from the template-displaying molecules, simply by sedimenting the beads in a bench centrifuge. Alternatively, the beads can be used to make an affinity column and the template-displaying libraries suspension recirculated through the column. There are many reactive matrices available for immobilizing the target molecule, including for instance attachment to $-NH_2$ groups and -SH groups. Magnetic beads are essentially a variant on the above; the target is attached to magnetic beads which are then used in the selection. Activated beads are available with attachment sites for $-NH_2$ or -COOH groups (which can be used for coupling). The target can be also be blotted onto nitrocellulose or PVDF. When using a blotting strategy, it is important to make sure the strip of blot used is blocked after immobilization of the target (*e.g.* with BSA or similar protein).

In another preferred embodiment, the selection or partitioning can also be performed using for example: Immunoprecipitation or indirect immunoprecipitation were the target molecule is captured together with template-displaying binding molecules; affinity column chromatography were the target is immobilized on a column and the template-displaying libraries are flowed through to capture target-binding molecules; gel-shift (agarose or polyacrylamide) were

the selected template-displaying molecules migrate together with the target in the gel; FACS sorting to localize cells that coordinates template-displaying molecules; CsCl gradient centrifugation to isolate the target molecule together template-displaying binding molecules; Mass spectroscopy to identify target molecules which are labelled with template-displaying molecules; etc., without limitation. In general, any method where the template-displaying molecule/target complex can be separated from template-displaying molecules not bound to the target is useful.

Table 1: Examples of selection method possible to use to identify binding molecules using the template-displaying technology.

| Type of Target | Method of choice |
|---|---|
| Soluble receptors | Direct immobilization, Immunoprecipitation, affinity column, FACS sorting, MS. |
| Cell surface receptor | Cell-surface subtraction selection, FACS sorting, Affinity column. |
| Enzyme inhibitors | Direct immobilization, Immunoprecipitation, affinity column, FACS sorting, MS. |
| Surface epitopes | Cell-surface subtraction selection, in-vivo selection, FACS sorting, Affinity column. |

Elution of template-displayed molecules can be performed in different ways. The binding molecules can be released from the target molecule by denaturation, acid, or chaotropic salts and then transferred to another vial for amplification. Alternatively, the elution can be more specific to reduce the background. Elution can be accomplished using proteolysis to cleave a linker between the target and the immobilizing surface or between the displaying molecule and the template. Also, elution can be accomplished by competition with a known ligand. Alternatively, the PCR reaction can be performed directly in the washed wells at the end of the selection reaction.

A possible feature of the invention is the fact that the binding molecules need not be elutable from the target to be selectable since only the encoding template DNA is needed for further amplification or cloning, not the binding molecule itself. It is known that some selection procedure can bind the most avid ligands so tightly as to be very difficult to elute. However the method of the invention can successfully be practiced to yield avid ligands, even covalent binding ligands.

Alternative selection protocol includes a known ligand as fragment of each displayed molecule in the library. That known ligand will guide the selection by coordinate to a defined part on the target molecule and focus the selection to molecules that binds to the same region. This could be especially useful for increasing the affinity for a ligand with a desired biological function but with a too low potency.

A further aspect of the present invention relates to methods of increasing the diversity or complexity of a single or a mixture of selected binding molecules. After the initial selection, the enriched molecules can be altered to further increase the chemical diversity or complexity of the displayed molecules. This can be performed using various methods known to the art. For example, using synthesized randomized oligonucleotides, spiked oligonucleotides or random mutagenesis. The randomization can be focused to allow preferable codons or localized to a predetermined portion or sub-sequence of the template nucleotide sequence. Other preferable method is to recombine templates coding for the binding molecules in a similar manner as DNA shuffling is used on homologous genes for proteins (Stemmer (1994) Nature 370:389-91). This approach can be used to recombine initial libraries or more preferably to recombine enriched encoding templates.

In another embodiment of the invention when binding molecules against specific antigens that is only possible to express on a cell surface, *e.g.* ion channels or transmembrane receptors, is required, the cells particle themselves

can be used as the selection agent. In this sort of approach, cells lacking the specific target should be used to do one or more rounds of negative selection or be present in large excess in the selection process. Here, irrelevant template-displayed molecules are removed. For example, for a positive selection against a receptor expressed on whole cells, the negative selection would be against the untransformed cells. This approach is also called subtraction selection and has successfully been used for phage display on antibody libraries (Hoogenboom et al. (1998) Immunotech. 4: 1-20).

A specific example of a selection procedure can involve selection against cell surface receptors that become internalized from the membrane so that the receptor together with the selected binding molecule can make its way into the cell cytoplasm or cell nucleus. Depending on the dissociation rate constant for specific selected binding molecules, these molecules largely reside after uptake in either the cytoplasm or the nucleus.

The skilled person in the art will acknowledge that the selection process can be performed in any setup where the target is used as the bait onto which the template-displaying molecules can coordinate.

The selection methods of the present invention can be combined with secondary selection or screening to identify molecule ligands capable of modifying target molecule function upon binding. Thus, the methods described herein can be employed to isolate or produce binding molecules which bind to and modify the function of any protein or nucleic acid. It is contemplated that the method of the present invention can be employed to identify, isolate or produce binding molecules which will affect catalytic activity of target enzymes, *i.e.*, inhibit catalysis or modifying substrate binding, affect the functionality of protein receptors, *i.e.*, inhibit binding to receptors or modify the specificity of binding to receptors; affect the formation of protein multimers, *i.e.*, disrupt quaternary structure of protein subunits; and modify transport properties of protein, *i.e.*, disrupt transport of small molecules or ions by proteins.

A still further aspect of the present invention relates to methods allowing functionality in the selection process can also be included. For example, when enrichment against a certain target have been performed generation a number of different hits, these hits can then directly be tested for functionality (*e.g.* cell signalling). This can for example be performed using fluorescence-activated cell sorting (FACS).

The altered phenotype may be detected in a wide variety of ways. Generally, the changed phenotype is detected using, for example: microscopic analysis of cell morphology; standard cell viability assays, including both increased cell death and increased cell viability; standard labelling assays such as fluorometric indicator assays for the presence of level of particular cell or molecule, including FACS or other dye staining techniques; biochemical detection of the expression of target compounds after killing the cells; etc. In some cases, specific signalling pathways can be probed using various reporter gene constructs.

Secondary selection methods that can be combined with template-displaying molecule technology include among others selections or screens for enzyme inhibition, alteration or substrate binding, loss of functionality, disruption of structure, etc. Those of ordinary skill in the art are able to select among various alternatives of selection or screening methods that are compatible with the methods described herein.

The binding molecules of the invention can be selected for other properties in addition to binding, For example, during selection; stability to certain conditions of the desired working environment of the end product can be included as a selection criterion. If binding molecules which are stable in the presence of a certain protease is desired, that protease can be part of the buffer medium used during selection. Similarly, the selection can also be performed in serum or cell extracts or any type of media. As will be understood, when util-

izing this template-displaying approach, conditions which disrupt or degrade the template should be avoided to allow amplification. Other desired properties can be incorporated, directly into the displaying molecules as will be understood by those skilled in the art. For example, membrane affinity can be included as a property by employing building blocks with high hydrophobicity.

Molecules selected by the template-displaying molecule technology can be produced by various synthetic methods. Chemical synthesis can be accomplished since the structure of selected binding molecules is readily obtained form the nucleic acid sequence of the coding template. Chemical synthesis of the selected molecules is also possible because the building blocks that compose the binding molecules are also known in addition to the chemical reactions that assemble them together.

In a preferred embodiment, the selected binding molecules is synthesized and tested in various appropriate *in vitro* and *in vivo* testing to verify the selected candidates for biological effects and potency. This may be done in a variety of ways, as will be appreciated by those in the art, and may depend on the composition of the bioactive molecule.

*Target identification and validation*

In another aspect, the present invention provides methods to identify or isolate targets that are involved in pathological processes or other biological events. In this aspect, the target molecules are again preferably proteins or nucleic acids, but can also include, among others, carbohydrates and various molecules to which specific molecule ligand binding can be achieved. In principal, the template-displaying molecule technology could be used to select for specific epitopes on antigens found on cells, tissues or *in vivo*. These epitopes might belong to a target that is involved in important biological events. In addition, these epitopes might also be involved in the biological function of the target.

Phage display with antibodies and peptide libraries has been used numerous times successfully in identifying new cellular antigens. (*e.g.* Pasqualini et al. (1996) Nature 380: 364-366; Pasqualini et al. (2000) Cancer Res. 60: 722-727; Scheffer et al. (2002) Br J Cancer 86: 954-962; Kupsch et al. (1999) Clin Cancer Res. 5: 925-931; Tseng-Law et al. (1999) Exp. Hematol. 27: 936-945; Gevorkian et al. (1998) Clin. Immunol. Immunopathol. 86: 305-309). Especially effective have been selection directly on cells suspected to express cell-specific antigens. Importantly, when selecting for cell-surface antigen, the template molecule can be maintained outside the cell. This will increase the probability that the template molecule will be intact after release for the cell surface.

In vivo selection of template-displayed molecules has tremendous potential. By selecting from libraries of template-displayed molecules in vivo it is possible to isolate molecules capable of homing specifically to normal tissues and other pathological tissues (*e.g.* tumours). This principle has been illustrated using phage display of peptide libraries (Pasqualini & Ruoslathi (1996) Nature 280: 364-366). This system has also been used in humans to identify peptide motifs that localized to different organs (Arap et al. (2002) Nat. Med. 2:121-127). A similar selection procedure could be used for the template-displaying libraries. The coding DNA in phage display is protected effectively by the phage particle allows selection *in vivo*. Accordingly, the stability of the template *in vivo* will be important for amplification and identification. The template can be stabilised using various nucleotide derivatives in a similar way as have been used to stabilise aptamers for in vivo applications (Nolte (1996) Nature Biotechnol. 14: 1116-1121; Pagratis et al. (1997) Nature Biotechnol. 15: 68-72). However, it is reasonable to believe that the template structure will be stabilized against degradation due to the modified bases used for encoding the displayed molecule. Other types of protection are also possible where the template molecule is shielded for the solution using various methods. This could include for example liposomes, pegylation, binding proteins or other sorts of protection. The template molecule could also be

integrated into another designed structure that protects the template form external manipulation. Fort example, the linker can be design to be incorporated in vesicles to position the templates inside the vesicle and the displaying molecules on the outside. The arrangement will protect the template molecules from external manipulate and at the same time allow exposure of the displaying molecules to permit selection.

Most antibodies have a large concave binding area which requires to some degree protruding epitopes on the antigens. Also, the antibody molecule is a large macromolecule (150 KDa) which will sterically reduce the access for a number of different antigens (*e.g.* on a cell surface). The template-displaying technology should be able to access and recognize epitopes inaccessible to antibodies. The small binding molecules will be able to bind into active sites, grooves and other areas on an antigen. The coding template element is also smaller that an antibody which will increase the physical access of the template-binding molecule par. In addition, the diversity and complexity of the template-displaying molecule libraries will be much greater compare to peptide libraries. This will increase the possibility to find molecules that can coordinate to epitopes inaccessible to peptides due to inadequate chemistry. All together, the template-displaying molecule technology has the potential to identify novel antigens which is not possible to identify with antibodies or peptides. One of ordinary skill in the art will acknowledge that various types of cells can be used in the selection procedure. It will also be understood that the selection for new antigens can be performed using subtraction methods as described previously.

Another aspect of the present invention relates to methods to validate the identified target. The identified binding molecules can directly be used if they change the biological response of the target. This can be done either *in vitro* using any direct or cell-based assay or directly *in vivo* studying any phenotypic response. The strength of this approach is that the same molecules are

used both for identification and validation of various targets. Most favourable, the binding molecules could also directly be used as therapeutic agents.

In another preferred embodiment, the template-displaying molecules are used to pull out the target molecules. This can for instance be achieved by selection against a cDNA library expressed on bacteriophage (libraries vs. libraries). By mixing a template-displaying molecule library with a cDNA library it will be possible to find binding pairs between the small molecules in the template-displaying molecule library and proteins from the cDNA library. One possibility is to mix a phage display library with a template display library and do a selection for either the phage or template library. The selected library is then plated to localized phage clones and the DNA coding for the phage and template displayed molecules can then be identified using PCR. Other types of libraries than cDNA could also be used such as nucleic acids, carbohydrates, synthetic polymer.

In another embodiment of the invention the template-displaying molecule technology can be used to account for in vivo and in vitro drug metabolism. That could include both phase I (activation) and phase II (detoxification) reactions. The major classes of reactions are oxidation, reduction, and hydrolysis. Other enzymes catalyze conjugations. These enzymes could be used as targets in a selection process to eliminate displayed molecule that are prone to coordinate to these enzymes. The templates corresponding to these displayed molecules could subsequently be used to compete or eliminate these molecules when making template-displaying molecule libraries. These obtained libraries will then be free of molecules that will have a tendency of binding to enzymes involved in phase I-II and possible be faster eliminated. For instance, a selection on each separate enzyme or any combination of cytochrome P450 enzymes, flavin monooxygenase, monoamine oxidase, esterases, amidases, hydrolases, reductases, dehydrogenases, oxidases UDP-glucuronosyltransferases, glutathione S-transferases as well as other relevant enzymes could be performed to identify these binding molecules that

are prone to coordinate to these metabolic enzymes. Inhibitors are easily selected for due to their binding affinity but substrates need at least micro molar affinity to be identified.

Another interesting embodiment of this invention is the possibility to directly select for molecules that passively or actively becomes transported across epithelial plasma membrane, or other membranes. One possible selection assay is to use CaCO-2 cells, a human colon epithelial cell line, which is general, accepted as a good model for the epithelial barrier in the gastrointestinal guts. The CaCO-2 assay involves growing a human colon epithelial cell line on tissue culture well inserts, such that the resultant monolayer forms a biological barrier between apical and basolateral compartments. The template-displaying molecule libraries are placed either side of the cell monolayer and the molecules that can permeate the cell monolayer is collected and amplified. This process can be repeated until active molecules have been identified. Other cell line or setup of this assay is possible and is obvious for skill in the art.

A still further aspect of the present invention relates methods of selecting for stability of the selected molecules. This could be performed by subjecting an enriched pool of binding molecules to an environment that will possibly degrade or change the structure of the binding molecules. Various conditions could be certain proteases or a mixture of protease, cell extract, and various fluids from for example the gastrointestinal gut. Other conditions could be various salts or acid milieu or elevated temperature. Another possibility is to generate a library of known ligands and subject that library to stability tests and selection to identify stable molecules under certain conditions as describe above.

*Therapeutic applications*

The potential therapeutic applications of the invention are great. For example, the template-displaying molecule technology of the invention may be

used for blocking or stimulating various targets. A therapeutically relevant target is a substance that is known or suspected to be involved in a regulating process that is malfunctioning and thus leads to a disease state. Examples of such processes are receptor-ligand interaction, transcription-DNA interaction, and cell-cell interaction involving adhesion molecules, cofactor-enzyme interaction, and protein-protein interaction in intracellular signalling. Target molecule means any compound of interest for which a molecule ligand is desired. Thus, target can, for example, include a chemical compound, a mixture of chemical compounds, an array of spatially localized compounds, a biological macromolecule, such as DNA or mRNA, a bacteriophage peptide display library, a ribosome peptide display library, an extract made from biological materials such as bacteria, plants, fungi, or animal (*e.g.* mammalian) cells or tissue, protein, fusion protein, peptide, enzyme, receptor, receptor ligand, hormone, antigen, antibody, drug, dye, growth factor, lipid, substrate, toxin, virus, or the like etc., without limitation. Other examples of targets include, *e.g.* a whole cell, a whole tissue, a mixture of related or unrelated proteins, a mixture of viruses or bacterial strains or the like. etc., without limitation.

Therapeutic drug targets can be divided into different classes according to function; receptors, enzymes, hormones, transcription factors, ion channels, nuclear receptors, DNA, (Drews, J. (2000) Science 287:1960-1964). Among those, receptors, nuclear receptors, and metabolic enzymes constitute overwhelmingly the majority of known targets for existing drugs. Especially, G Protein-Coupled Receptors (GPCR) constitutes one of the most important classes of drug targets together with proteases for pharmacological intervention. Although the above examples are focused on the most relevant targets, it will be self-evident for a person skilled in the art that any other therapeutic target may be of interest.

The present invention employing the template-displaying molecule technology can be utilized to identify agonists or antagonists for all these classes of

drug targets, dependent on the specific properties each target holds. Most of the targets are possible to obtain in a purified form for direct selection procedures. Other targets have to be used when they are in their native environments such as imbedded cell surface receptors. In those situations the selection using the template-displaying molecule libraries can be performed using subtraction-selection described previously.

One specific application of the template-displaying molecule technology of the invention is to generate molecules that can function as antagonists, where the molecules block the interaction between a receptor and one or more ligands. Another application includes cell targeting. For example, the generated molecules recognizing specific surface proteins or receptors will be able to bind to certain cell types. Such molecules may in addition carry another therapeutic agent to increase the potency and reduce the side-effects (for example cancer treatment). Applications involving antiviral agents are also included. For example, a generated molecule, which binds strongly to epitopes on the virus particle, may be useful as an antiviral agent.
Another specific application of the template-displaying molecule technology of the invention is to generate molecules that can function as agonists, where the molecules stimulate or activate a receptor to initiate a cellular signalling pathway.

**Brief description of the figures**

Fig. 1 shows the general principle for one embodiment of the present invention for the multi-step synthesis of templated molecules.

Fig. 2 shows the general structure of templates useful in the generation of a library.

Fig. 3 shows an example of design of templates fro the generation of a library.

Fig. 4 shows examples of building blocks for use in the preparation of a library of templated molecules.

Fig. 5 shows further examples of building blocks.

Fig. 6 shows examples of the preparation of building blocks.

Fig. 7 shows examples of the preparation of building blocks starting from a 5'–$NH_2$ derivatized oligonucleotide.

Fig. 8 shows a general procedure of performing one embodiment for the formation of the templated molecule.

Fig. 8, example 1, shows an example of the embodiment shown in Fig. 8 involving light induced reaction between symmetrical building blocks.

Fig. 9 shows a general procedure of performing one embodiment for the formation of the templated molecule.

Fig. 10 shows a general procedure of performing one embodiment of the invention for the formation of a mixed polymer templated molecule.

Fig. 10, example 1, shows examples of simultaneous reaction and cleavage of neighbouring of functional entities for the formation of (A) and alpha-peptide and (C) a polyamine..

Fig. 10, example 2, shows examples of simultaneous reaction and cleavage of neighbouring functional entities for the formation of (A) a peptoid, or an α- or β-peptide, and (B) a hydrazino peptide.

Fig. 11 depicts a templated synthesis of a polymer, using non-simultaneous reaction and cleavage.

Fig. 12 depicts formation of a templated molecule due to activation of reactive groups and partly release of the templated molecule for the template by ring-opening.

Fig. 13 shows the connection of two functional entities by the fill-in of connecting moiety.

Fig. 13, example 1, discloses an exemplification of Fig. 13, in which an imine is formed by a fill-in reaction.

Fig. 13, example 2, shows an exemplification of Fig. 13, in which an amide is formed.

Fig. 13, example 3, shows an exemplification of Fig. 13, in which an urea bonding is formed.

Fig. 13, example 3.1, shows an exemplification of Fig. 13 in which functional entities 13.3.1.A and 13.3.1.B are synthesised.

Fig. 13, example 4, shows the formation of chiral and achiral templated molecules.

Fig. 13, example 5, shows the formation of a phosphodiester bond by symmetric fill-in.

Fig. 13, example 6, shows the formation of a phosphodiester bond by a fill-in reaction, wherein the building block comprises a single reactive group.

Fig. 13, example 7, shows a pericyclic fill-in reaction.

Fig. 13, example 7.1, shows an exemplification of Fig. 13 in which functional entities 13.7.1.A and 13.7.1.B are synthesised.

Fig 14 shows a schematic representation of a fill-in reaction using asymmetric monomers.

Fig. 14, example 1, shows an asymmetric fill-in reaction using modified Staudinger ligation and ketone-hydrazide reaction.

Fig. 15 shows a schematic representation of a templated synthesis of a non-linear molecule.

Fig. 16 shows a representation of the templated synthesis of a non-linear molecule employing reactive groups of different classes and non-simultaneous reaction and cleavage.

Fig. 17 depicts a templated synthesis of a non-linear molecule, by exploiting the increased proximity effect that arises from a "migrating" scaffold.

Fig. 18 shows examples of the templated synthesis of non-linear molecules.

Fig. 19 shows a schematic representation of a templated synthesis, wherein the reaction step may be performed under conditions where specific annealing of building blocks to the template is inefficient.

Fig. 20 shows examples of various reactions types allowing simultaneous reaction and cleavage.

Fig. 21 shows examples of pairs of reactive groups (X) and (Y), and the resulting bond (XY).

Fig. 22 shows a schematic representation (panel A) of the zipper box principle and an example (panel B) of two building block.

Fig. 23 shows a schematic representation of various methods for increasing the proximity of functional entities of different building blocks.

Fig. 24 shows examples of the chemical constitution of a linker to be able to be cleaved.

Fig. 25 schematically shows the templated synthesis by generating a new reactive group.

Fig. 25, example 1, shows a method in which reactive groups generated in a first round subsequently are reacted with introduced reactive groups.

Fig. 26 shows examples of post-templating modifications of the templated molecule.

**Detailed description of the invention**

The following symbols are used in the figures to indicate general characteristics of the system: In figures 8; 10; 10, examples 1 and 2; 12; 12, examples 1; 13; 13, examples 1-7; 14; 14, example 1; 18; 20; 22; 23; 25; 25, example 1, a long horizontal line symbolizes a template. Coding region 1 symbolizes sequences that anneal to type 1 building blocks. Building blocks are symbolized as shown in figures 4-7. X/Y, S/T and P/Q represent pairs of reactive groups (where the reactive groups of one pair (e.g. X and Y) are partly or fully orthogonal to the reactive groups of other pairs (e.g. S/T, P/Q)). $R_1$, $R_2$, .., $R_x$ symbolize functional groups. L and $L_1$, $L_2$, $L_3$,... represent cleavable linkers, where linkers of one group (e.g., $L_1$-linkers) are cleavable under conditions where linkers of other groups ($L_2$, $L_3$, ....) are not cleaved, or are cleaved less efficiently. The proximity effect that results from incorporating two building blocks on the same template, or alternatively, as a result of incorporating a building block on a template to which is attached a reactive group, may be enhanced by any of the methods described above or below that increases this effect. For example, in order to increase the efficiency and specificity of templated synthesis, the proximity effect may be increased by the introduction of zipper boxes in most of the general concepts described here.

In all the examples, the templated molecule may be coupled to the template through the non-covalent interaction of a monomer building block with the

template, or alternatively, through covalent or non-covalent coupling to the template, and may be located at either of the ends of the template, or anywhere on the template. The coupling reaction to the template may be performed before, during or after the synthesis of the templated molecule. For clarity, in some of the figures only the reaction step, not the cleavage step, has been included.

The figures included have been drawn so as to highlight specific set-ups. Obviously, any combination of the methods may be employed, in order to make linear, as well as non-linear molecules, to use reactive groups that lead to simultaneous cleavage, as well as reactive groups that do not lead to simultaneous cleavage, to use cleavable and non-cleavable linkers etc.

The protocol for an embodiment of a multi-step templated synthesis is shown in Fig. 1 and involves a number of steps that each result in the addition of one or more molecular moieties to a growing molecule that eventually becomes the templated molecule. Each of these steps can be divided into substeps. Initially, a number of templates (also called a library of templates) are provided. Each of the templates comprises a plurality of unique codons and a reactive group. Also, a plurality of different building blocks are provided, each of the building blocks comprises a functional entity separated from an anti-codon with a suitable linker. The anti-codon of a specific building block complements a unique codon of a template and is, therefore, capable under proper hybridisation conditions to anneal to the unique codon. The incorporation of building blocks is initiated by contacting the plurality of different templates with a subset of the entire amount of building blocks. The subset carries anti-codons which hybridise to unique codons of a distinct coding region. A connection between the reactive group of the template and the functional entity of the building blocks is obtained. In Fig. 1 the reactive group of the template is part of a building block (building block 1) and the said building block is hybridised to the template. In a preferred embodiment the building block 1 comprising the reactive group of the template and the second build-

ing block are contacted with the template simultaneously to allow for an efficient connection between the functional entities. The line between $FE_1$ and $FE_2$ symbolise a direct connection between the functional entities or an indirect connection via a bridging molecule entity. The molecule part formed by a connection of $FE_1$ to $FE_2$ is a nascent templated molecule, which may be added further functional entities resulting in a growing nascent templated molecule.

The propagation part of the method starts with the incorporation of a further building block (building block 3). The incorporation involves the hybridisation of a subset of the building blocks to the plurality of templates bearing the nascent templated molecule. The subset of building blocks is selected to have anti-codons which complement unique codons of the templates, said unique codons being in the vicinity of, preferably neighbouring to, unique codons hybridised to the building block(s) bearing the templated molecule. The functional entity of the further building block is able to form a chemical connection to the nascent templated molecule through the reaction of a reactive group attached to the functional entity. The linkage between one or more of the functional entities and the corresponding anti-codons may be cleaved if desired and the incorporation of a new building block may be performed. In the example illustrated in Fig. 1 only three functional entities are connected in the templated molecule. However the propagation step may be conducted as many times as appropriate to obtain the desired templated compound.

As a terminal phase the linkers connecting functional entities/templated molecule and anti-codons may be cleaved. The complex comprising the templated molecules (specific compositions or sequences of molecular moieties, the identity of which is determined by the template) attached to the templates that templated their synthesis, can now be taken through a screening process. This process leads to an enrichment of templated molecules complexes with appropriate characteristics. The isolated complexes may now be enriched by amplification of the templates, and a new round of templated

synthesis and screening can be performed. Eventually, the templated molecules may be identified by characterization of the corresponding templates.

The stages of the process involving incorporation of building blocks may be mediated by chemicals, or enzymes such as polymerase or ligase. For example, the anti-codon part of the building blocks may be nucleotide-derivatives that are incorporated by a polymerase. Incorporation may also be solely by hybridization of building blocks to the template. If the template is a DNA molecule, the template may comprise primer binding sites at one or both ends (allowing the amplification of the template by e.g. PCR). The remaining portion of the templates may be of random or partly random sequence.

The reaction stage of the method involves reactions between the incorporated building blocks, thereby forming chemical connections between the functional entities. The chemical connection can be a direct chemical bond or the connection can be established through a suitable bridging molecule.

The optional cleavage step involves cleaving some, all but one, or all of the linkers that connect the functional entities and anti-codons. In Fig. 1 the templated molecule is displayed by cleaving the linkers of the second and third functional entities, while maintaining the linker from the first building block.

Subsequent to the production of library according to the invention a selection is performed. The selection or screening involves enriching the population of template-templated molecule pairs for a desired property. For example, passing a library of templated molecule-template complexes over a solid phase to which a protein target has been immobilized, and washing unbound complexes off, will enrich for complexes that are able to bind to the protein.

The selection may be performed more than once, for example with increasing stringency. Between each selection it is in general preferable to perform an amplification. The amplification involves producing more of the template-templated molecule complexes, by amplification of the template or complementing template, and producing more of the template-templated molecule pairs, for further rounds of selection/screening, or for sequencing or other characterization. For example, if the template is a DNA strand, the template may be amplified by PCR, where after the templated synthesis can be performed using the amplified DNA, as described above.

Cloning and sequencing may also be useful techniques and involve the cloning of the isolated templates or complementing templates, followed by characterization. In some cases, it may be desirable to sequence the population of isolated templates or complementing templates, wherefore cloning of individual sequences is not required.

In Fig. 2, in the upper part of the figure, the general structure of a template is shown. The templates comprise x coding regions. Each coding region has a unique sub-structure which differentiates it from some or all of the other coding regions. Shown below the general structure of a template are specific templates. A given specific template carries a specific set of x unique codons. A unique codon specifies (by way of interaction with a specific anti-codon of a building block) a specific functional entity. The unique codons 1.1, 1.2, 1.3, ....., 1.m are all examples of coding region 1 sequences. The general design of the templates therefore enables the templated incorporation of building blocks, in the sense that a sub-set of building blocks can be added that will only be incorporated at the same position on the template (i.e., coding region 1 if the building blocks have anti-codons that are complementary to the unique codons of codon region 1).

Fig. 3 Shows an example of a design of templates and anti-codons for oligonucleotide-based building blocks. Section A discloses the general

structure of a set of templates carrying 6 coding regions, each containing a partly random sequence (X specifies either C or G), and a constant sequence that is identical for all sequences in the group (e.g., all coding region 1 sequences carries a central ATATTT sequence). By using C and G only (or, alternatively, A and T only), the building blocks that are complementary to coding regions 1 have very similar annealing temperatures wherefore mis-annealing is insignificant. The attachment point of the linker that connects the anti-codon and the functional entity is not specified in the figure. Ideally, the linker is attached to the constant region of the anti-codon, in order to avoid bias in the annealing process.

Section B of Fig. 3 shows examples of codon and anti-codon sequences. Example codon 1 and codon 6 sequences are shown. The example codon 1 sequence represents one specific sequence out of 1024 different sequences that anneal specifically to the complementary anti-codon 1 sequences; the example codon 6 sequence represents one specific sequence out of 128 different sequences that anneal to the complementary anti-codon 6 sequences.

Fig. 4 illustrates different general designs of building blocks. A building block comprises or essentially consists of a functional entity, connected through a (cleavable) linker to an anti-codon. Panel A shows a building block with one reactive group (X), connecting the functional group ($R_x$) with the anti-codon. This type of building block may be used for the simultaneous reaction and cleavage protocol (e.g. figures 9 and 15). The functional entity in this example comprises one reactive group, and a functional group $R_x$, also called a functionality. The reactive groups typically become part of the templated molecule. Panel B shows a building block with two reactive groups (X and Y), connecting the anti-codon and the functional group ($R_x$). The functional entity in this example comprises two reactive groups that are both part of the moiety that links the anti-codon and functional group, $R_x$. Panel C shows a building block with a reactive group (X) connecting $R_x$ and the anti-codon, and a

reactive group (Y) attached to the $R_x$ group. This type of building block may be used in the simultaneous reaction and cleavage protocol (e.g., Fig. 9 and 10). The functional entity comprises two reactive groups X and Y, where X is part of the linker, and Y is attached to the functional group $R_x$. Panel D shows a building block with one reactive group (X). The reactive group (X) does not link the functional group (Rx) and the complementing element. A cleavable linker (L) is provided in order to release the functional entity from the anti-codon. This type of building block may be used in protocols that require cleavage of the linker after the reactive groups of the functional entities have reacted (e.g., Fig. 12). Panel E disclose a building block with four reactive groups and a functional group Rx. The four reactive groups and the functional group $R_x$ may serve as a scaffold, onto which substituents (encoded by building blocks bound to codons on the same template) are coupled through reaction of reactive groups (X) of other building blocks with the reactive groups (Y) (e.g., figure 15). In this example, no cleavable linker is indicated. Therefore, after the templating reactions the templated molecule is attached to the template through the linker of this building block.

In Fig. 5 three different building blocks are depicted. Building block A comprises an anti-codon (horizontal line), which may be an oligonucleotide, to which a linker carrying the functional entity is attached to the central part. The portion of the linker marked "a" may represent a oligonucleotide sequence to which a single stranded nucleotide may be annealed in order to make the linker more rigid, or alternatively, "a" may represent a zipper box sequence of nucleotides or other type of zipper box moiety. The vertical line may represent a PEG (polyethylene glycol) linker, oligonucleotide linker, or any other linker that provides the functional entity with the appropriate freedom interact productively with a functional entity of a building block annealed to the same template during the templating process. In building block, the linker is attached to the terminus of the anti-codon. The anti-codon and the linker may be one continuous strand of an oligonucleotide. The horizontal part here represents the anti-codon, and the vertical part

represents the linker. The linker may contain a moiety "a" that functions as a zipper box (see Fig. 22), a rigid linker, or an annealing site for another entity that rigidifies the linker upon annealing. In building block C of Fig . 5 the linker and anti-codon may be a continuous strand of an oligonucleotide. Attached to the linker is a nucleophile "Nu" which may react with a functional entity. This may be used as an anchorage point for the templated molecule. Building block C may preferably be used as the starting or the terminal building block. When used in the initial stage of the production of the complex comprising the templated molecule, building block C may provide the template with a reactive group to which the functional entities may be attached in the growing templated molecule. In a further embodiment of the invention "Nu" of building block C represents any reactive group able to participate in a reaction resulting in the formation of a connection to a functional entity of a building block.

Fig. 6 shows five different general methods for the preparation of building blocks. The general methods involves the coupling of the functional entities to oligonucleotide-based building blocks. Reactions and reagents are shown that may be used for the coupling of functional entities to modified oligonucleotides (modified with thiol, carboxylic acid, halide, or amine), without significant reaction with the unmodified part of the oligonucleotide. As an alternative approach, the functional entity may be synthesized as phosphoramidite precursor, which can then be used for oligonucleotide synthesis by standard methods, resulting in an oligonucleotide-derivative carrying a functional entity.

Fig. 7 shows the design and synthesis of exemplary building blocks. Panel A shows a general synthesis scheme for building blocks using DNA oligonucleotide as codon, and coupling amines and carboxylic esters. The oligonucleotide is purchased with an amine coupled to e.g. the base at a terminal position of the oligo. By addition of EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) and NHS (N-

hydroxysuccinimide), the oligonucleotide is coupled to the building block through an amide bond. Panel B shows specific synthesis schemes for the generation of specific classes of building blocks.

Fig. 8 illustrates an embodiment for the templated synthesis of a polymer. X and Y are reactive groups of the functional entity. X and Y may be different kinds of reactive groups (e.g., amine and carboxylic acid), of the same kind but different (e.g., different primary amines or a primary amine and a secondary amine), or identical. Reaction of X with Y to form XY either happens spontaneously when the building block has been incorporated, or is induced by a change of conditions (e.g. pH), or by the addition of an inducing factor (chemical or UV exposure, for example)

Fig. 8, ex.1. shows light-induced reaction between symmetric coumarin-derivatives. Light-induced reaction of the coumarin units, followed by cleavage of the linker, result in a ring structure. Examples of functional groups (phosphate and carboxylic acid) are shown. The building blocks are said to be symmetric because the two reactive groups, two coumarin units, are of the same reactivity (in fact, in this example are identical).

Fig. 9 shows an embodiment for templated synthesis of a polymer. A population of templates, each carrying four codons are incubated with two sets of building blocks (carrying anti-codons 1 and 2, respectively), at a temperature that ensures efficient and specific annealing of anti-codons type 1 to coding regions 1, and efficient and specific annealing of anti-codons type 2 to coding regions 2. After annealing, the excess building blocks may optionally be removed. If desired, reactive groups may be deprotected (and thus activated for reaction) at this step. Then building block-template complexes are incubated under conditions that allow the reactive groups of the building blocks (i.e., reactive groups X and Y) to react. This leads to a transfer of the functional group R1 from building block 1 to building block 2, and thus results in the formation of a dimeric polymer carrying two functional

groups, R1 and R2. The process is then repeated, i.e. a third monomer (with anti-codon type 3) is added, and after annealing to coding region 3, excess building block is removed, and the reaction between X and Y now leads to the formation of a trimeric polymer, coupled to the building block annealed to coding region 3. Once more, the process is repeated with building blocks of type 4, resulting in the formation of a tetrameric polymer.

The reactive groups X and Y used in this scheme thus have two functions: i) reaction between X and Y leads to coupling of the corresponding functionalities (e.g., R1 and R2), and simultaneously, ii) the linker between R1 and the anti-codon is cleaved. Examples of reactive groups X and Y with such characteristics (i.e., the ability to simultaneously react and cleave) are shown in Fig. 20. By appropriate choice of X and Y, the nascent polymer is migrated down the template, from building block to building block, as it is being synthesized. For example, by choosing X = ester (COOR), and Y = amine ($NH_2$), the nucleophilic attack of the amine on the ester leads to transfer of the upstream functionality (e.g., $R_1$) to the downstream building block (e.g., carrying anti-codon type 2). This ensures the highest possible effect of proximity with this set-up (i.e., in each step, the reacting X and Y are carried on neighbouring monomers).

If desired, the templated polymer may be coupled to the template through the non-covalent interaction of a building block with the template (in the example given, through the interaction of building block 4 with the template), or alternatively, through covalent coupling to a reactive group on the template, located at either of the ends of the template, or anywhere on the template sequence. In the latter case, the coupling reaction to the template may be performed before, during or after the synthesis of the polymer.

Fig. 10 shows the templated synthesis of a mixed polymer. The most noticeable difference, when compared to the embodiment shown in Fig. 9 is that the reactive groups on the individual building blocks are different. The

pairs of reactive groups (X/Y, S/T, and P/Q) are chosen so that the reaction of X and Y, S and T, P and Q, respectively, results in transfer of a functional group from one building block to another (i.e., the reaction both mediates the coupling of the two functional groups and the cleavage of the linker that initially connects one of the functional groups to the anti-codon). Example pairs of reactive groups that mediate this simultaneous reaction and cleavage are shown in Fig. 20.

Fig. 10, example 1 shows two methods of obtaining different classes of compounds using simultaneous reaction and cleavage. In panel A, the formation of an alpha-peptide is disclosed and in panel C the synthesis of a polyamine is shown.

In panel A, two building blocks are incorporated by hybridization to the template. One of the building blocks is an oligonucleotide to which has been appended a thioester. The other building block is an oligonucleotide to which has been appended an amino acid thioester. The amine of the latter building block attacks the carbonyl of the other building block. This results in formation of an amide bond, which extends the peptide one unit. When the next amino acid thioester building block is incorporated, this may attack the thioester carbonyl, resulting in cleavage of the dipeptide from the anti-codon, to form a tripeptide. This process is repeated until the desired peptide has been generated. Importantly, as the reaction in each step is between the incoming subunit-precursor and the subunit of the nascent polymer that is closest to the linker that connects it to the anti-codon, the geometry of the nucleophilic attack remains unchanged. The reactivity of the amine with the ester may be tuned in several ways. Parameters that will affect the reactivity include: (i) pH and temperature, (ii) nature of ester (thio-, phospho-, or hydroxy-ester); (iii) the nature of the substituent on the sulfur (see panel B below).

The general scheme presented here can be applied to most nucleophilic reactions, including formation of various types of peptides, amides, and amide-like polymers (e.g., mono-,di-, tri-, and tetra-substituted α-, β-, γ-, and Ω-peptides, polyesters, polycarbonate, polycarbamate, polyurea), using similar functional entities.

Panel B shows four different thioesters with different substituents on the sulphur and therefore different reactivity towards nucleophiles.

Panel C relates to the formation of a polyamine. Using the same principle as in (A), a polyamine is formed.

Fig. 10, example 2 shows simultaneous reaction and cleavage for two reactions. In reaction A a peptoid or an α- or β-peptide is formed, and in reaction B a hydrazino peptide is formed.

In reaction A, two building blocks are initially incorporated, one of which carries both a nucleophile (an amino group) and an electrophile (e.g. an ester); the other building block only carries an electrophile (e.g. a thioester). As a result, the nucleophilic amine will attack the electrophile of the building block attached to the same template. As a result, a dimeric structure is formed, linked to building block that initially carried the amine. Upon sequential addition of building blocks, the linear structure grows, and eventually the desired templated molecule (a peptoid or an α- or β-peptide) has been formed.

The reaction B follows the same line as in A, except that hydrazine-peptide precursor building blocks are used, leading to the formation of hydrazino peptides.

Fig. 11 shows a general reaction scheme for templated synthesis of a polymer, using non-simultaneous reaction and cleavage. In this scheme, the

reaction of the reactive groups (e.g., X and Y) does not in itself lead to cleavage, wherefore the functional entity is coupled to the anti-codon via a cleavable linker. Therefore, each addition of a subunit to the growing polymer involves two steps. First, the reactive groups X and Y react to form a bond XY. Then, in a separate step, a cleavable linker L is cleaved, which releases one of the functional entities from the anti-codon. By alternating between two types of cleavable linkers (cleavable under different conditions) one may achieve migration of the nascent polymer down the template, like described in fig. 9 and 10. This ensures the highest possible effect of proximity with this set-up (i.e., in each step, the reacting X and Y are carried on neighbouring monomers). In the example, some or all of the reactive pairs may be of the same kind (e.g., X/Y = S/T = P/Q).

Example reactions that do not mediate simultaneous reaction and cleavage are shown in fig. 21. Any combination of cleavable and non-cleavable linkers may be used, dependent on the nature of the reactive groups in the functional entities (e.g., dependent on whether the reaction involves a release from the anti-codon).

Fig. 12 relates to activation of reactive groups and release from anti-codon by ring-opening.
Reaction of the initiator with X in the ring structure opens the ring, resulting in activation of Y. Y can now react with X in a neighboring functional entity. As a result of ring-opening, the functional entities are released from the anti-codons.If the zipper-box principle is applied to this set-up (where each additional building block added reacts with the nascent templated molecule attached to the initiator), the initiator linker must carry half of the zipper (e.g., the "sense strand"), and all the building blocks must carry the other half of the zipper-box (the "anti-sense strand").

Fig. 12, example 1. Ring-opening of N-thiocarboxyanhydrides, to form β-peptides.

After incorporation of two building blocks, where one of the building blocks carry an initiator reactive group (or incorporation of one building block next to a covalently attached initiator molecule), the initiator is activated, for example by deprotection or by an increase in pH. The primary amine then attacks the carbonyl of the N-thiocarboxyanhydride (NTA) unit. As a result, CSO is released, and a primary amine is generated. When the next building block is incorporated, this amine will react with the NTA, and eventually when all the building blocks have been incorporated and the NTA units have reacted, a β-peptide will have formed. Finally, the linkers that connect the β-peptide to the anti-codons are cleaved, resulting in a β-peptide attached to its template through one linker.

A number of changes to this set-up can be envisaged. For example, instead of thiocarboxyanhydrides, one might use carboboxyanhydrides. The initiator might be protected with a base- or photolabile group. If a base-labile protection group is chosen, the stability of the carboxyanhydride must be considered. At higher pH it may be advantageous to use carboxyanhydrides rather than thiocarboxyanhydrides. Other types of peptides and peptide-like polymers (e.g., mono-,di-, tri-, and tetra-substituted α-, β-, γ-, and Ω-peptides, polyesters, polycarbonate, polycarbamate, polyurea) can be made, using a similar scheme. For example, α-peptides can be made by polymerization of 5-membered carboxyanhydride rings.

Fig. 13 shows the principle of symmetric fill-in (symmetric XX building blocks). The fill-in reaction occurs between the reactive groups ("X" in the figure) and bridging molecules "Y-Y" in figure).
For clarity, only the reaction (not the cleavage) is shown in the figure. X represents the reactive groups of the functional entity. In this case the two reactive groups are of the same kind. (Y-Y) is added to the mixture before, during or after incorporation of the building blocks.

Fig. 13, ex.1 shows imine formation by fill-in reaction.

Dialdehyde is added in excess to incorporated diamines. As a result, an imine is formed. In the example, the templated molecule carries the following functional groups: cyclopentadienyl and hydroxyl.

Fig. 13, example 2 shows an example of amide formation using symmetric fill-in. After incorporation of two building blocks each carrying a di-amine, non-incorporated building blocks may be removed. Then EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide), and dicarboxylic acid is added in excess to the primary amines of the building blocks. Alternatively, a di-(N-hydroxy-succinimide ester) may be added in excess. At a pH of 7-10, this will lead to the formation of two amide bonds linking the functional entities. After reaction, excess reagents may be removed by dialysis, precipitation of the building blocks and template, gel filtration or by other means that separate the reagents from the building blocks. When the process of incorporation-and-reaction has been repeated a number of times, and the desired molecule has been templated, the linkers (L) may be cleaved, and, if functional groups have been masked by protection groups (PG), these functional groups can be deprotected to expose the functional groups. Appropriate protecting groups would be for example Boc-, Fmoc, benzyloxycarbonyl (Z, cbz), trifluoracetyl, phthaloyl, or other amino protecting groups described e.g. in (T. W. Green and Peter G. M. Wuts (1991), Protective Groups in Organic Synthesis).

An alternative route to amide-bonded functional entities would be to incorporate building blocks carrying di-carboxylic acids, and then add di-amines, NHS and EDC. Alternatively, the building blocks could carry N-hydroxy-succinimidyl (NHS) esters, which would react with the added amines without the need to add EDC and NHS.

Fig. 13, example 3 shows an example of urea-bond formation.
The functional entities of the incorporated building blocks react with phosgen or a phosgen-equivalent such as CDI to form a polyurea. Formaldehyde may

also be used. The linkers are cleaved and the protected hydroxyl is deprotected. Appropriate leaving groups (Lv) are chloride, imidazole, nitrotriazole, or other good leaving groups.

Fig.13, example 4 shows the formation of chiral and achiral templated molecules. In this example, the functional group Rx is used as a cleavable linker, that generates the desired functional group upon activation. In both reaction A and reaction B, a urea-bond is formed.

In reaction A, the functional group is attached to the backbone via a chiral carbon. The hydrogen on this carbon is drawn to emphasize this. Before bond formation, there is free rotation about the bond connecting the chiral carbon and the functional group. When the reactive groups (the amines) react with the phosgen equivalent (e.g., a carbonyldiimidazole) to form the templated molecule, the building blocks may be inserted in either of two orientations (as indicated by the position of the hydrogen, left or right). As a result, each encoded residue of the templated molecule will have two possible chiral forms. If the templated molecule was e.g. a pentameric polyurea (formed from five functional entities), this molecule would have $2^5$ = 32 stereoisomers. In certain cases it may be advantageous to incorporate such additional structural diversity in the library (for example when a low building block diversity is employed). In other cases such additional diversity is not desirable, as the screening efficiency may become compromised, or it may become too difficult to determine the identity of a templated molecule that has been isolated in a screening process.

In reaction B, the chiral carbon of reaction A has been replaced by a nitrogen. As a result, the resulting templated molecule is achiral, i.e. the template encodes one specific structure.

Fig.13, example 5 shows the formation of a phosphodiester bond by the principle of symmetric fill-in. The incorporated building blocks react with the

activated fill-in molecule to form a phosphodiester bond. Then the linkers are cleaved, releasing the templated molecule from its template. An example of an appropriate leaving group (Lv) is imidazole.

Fig. 13, example 6 shows phosphodiester formation with one reactive group in each building block.
Upon addition of a dihydroxylated compound such as 1,3-dihydroxypyridine, a phosphodiester bond is formed. Finally, the functional group Rx is liberated from the anti-codon by cleavage of the protection groups/cleavable linker that connected it to the anti-codon.

Fig.13, example 7 shows the an example of a pericyclic fill-in reaction. First, two building blocks are incorporated. Then 1,4-benzoquinone is added in excess, resulting in the formation of a polycyclic compound. A third building block is added, reacted with the 1,4-benzoquinone, and this process is repeated a number of times until the desired templated molecule has been generated. Finally, all but one of the linkers that connect the templated molecule to the anti-codon, are cleaved.

Fig. 14 relates to asymmetric fill-in using XS building blocks.
A fill-in reaction between reactive groups (X and S) and bridging molecules (T-Y) is shown. For clarity, only the reaction (not linker cleavage) is shown. X and S represent the reactive groups of the functional entity. In this case the two reactive groups are not of the same kind. (T-Y) is added to the mixture before, during or after incorporation of the building blocks. Likewise, significant reaction between X and Y, and between S and T may take place during or after incorporation of the building blocks.

Fig. 14, example 1 shows an example of asymmetric fill-in by modified Staudinger ligation and ketone-hydrazide reaction. The reactive groups X and S of the building blocks are azide and hydrazide. The added molecule that fills the gaps between the building blocks carry a ketone and a

phosphine moiety. The reactions between a ketone and a hydrazide, and between an azide and a phosphine, are very chemoselective. Therefore, most functional groups Rx can be employed without the need for protection during the reactions. Examples for the molecular moieties R, R1, X and Y may be found in (Mahal et al. (1997), Science 276, pp. 1125-1128; Saxon et al. (2000), Organic Letters 2, pp. 2141-2143).

Fig. 15 shows a general reaction scheme for templated synthesis of a non-linear molecule. A template carrying four codons is mixed with two building blocks. The functional entity of one building block comprises a reactive group X and a functional group $R_1$. The other building block comprises three reactive groups Y and a functional group $R_2$. The building block bound to codon 2 is here called the scaffold, as the functional groups are transferred to this building block during the templating process.

After incubation at a temperature that ensures efficient and specific annealing of the two building blocks to their respective codon, and optionally, excess building block has been removed, X is brought to react with one of the reactive groups Y, for example by changing the conditions, by deprotecting X or Y, or by simply exploiting the pronounced proximity of X and Y groups when the building blocks are bound to the template.

In this scheme, X and Y have been chosen so as to allow simultaneous reaction and cleavage. Thus, as a result of the reaction between X and Y, the substituent group (functional group) $R_1$ is transferred to the scaffold. Example reactive groups X and Y that mediate simultaneous reaction and cleavage are given in figure 20. Any pair of reactive groups X and Y that mediates simultaneous reaction and cleavage can be used in this scheme, i.e., different X/Y pairs may be used at each substituent position.

Annealing and reacting of two more building blocks lead to the formation of a scaffolded molecule carrying three substituents ($R_1$, $R_3$ and $R_4$). The identity

of the substituents is determined by the codons of the template to which the scaffolded molecule is attached.

Figure 16 shows templated synthesis of a non-linear molecule, employing reactive groups of different kinds, and non-simultaneous reaction and cleavage. The reactive groups X, S, P and Y, T, Q may be of different kinds, and the bonds formed (XY, ST, and PQ) therefore may be of different kinds.

After reaction and then cleavage of the linker L (that attaches the functional entity of the first building block to the anti-codon), the substituent (functional group) R1 is transferred to the second building block (the scaffold). Thus, relative to the synthesis scheme of figure 15, here an additional step of linker cleavage is required. After repeating the processes of annealing, reacting and cleavage a number of times, a scaffolded molecule has been formed carrying encoded substituents. The identity of the substituents is determined by the codons of the template to which the scaffolded molecule is attached. The position of the substituents are determined by the identity of the reactive groups Y, T and Q of the scaffold, and therefore indirectly determined by the identity of the codon to which the scaffold building block anneals. Therefore, in this set-up, the identity and position of the substituents, as well as the identity of the scaffold, is determined by the sequence of the template. The reactive pairs may also be of the same kind (e.g., X/Y = S/T = P/Q).

Fig. 17 discloses the principle of templated synthesis of a non-linear molecule, by exploiting the increased proximity effect that arises from a "migrating" scaffold. In this set-up, the templated molecule migrates down the template as it is being synthesized. This is made possible by the use two different linkers $L_x$ and $L_y$, cleavable under different conditions. As a result, a high proximity is maintained throughout the templating process, as the building blocks that react in each reaction step are bound to adjacent coding regions on the template.

Fig. 18 shows the templated synthesis of various non-linear molecules.

(A) Three building blocks are added and reacted one at a time. Each building block comprises an activated ester (reactive group, (X)) where the ester moiety carries a functional group Rx. Upon reaction between the esters and the amines on the scaffold (scaffold is covalently attached to the template), amide bonds are formed, and the Rx groups are now coupled to the scaffold via amide bonds. This is thus an example of simultaneous reaction (amide formation) and cleavage (release of the Rx moiety from the anti-codon), see e.g. fig.15.

(B) Analogously to (A), three amines react with three esters to form three amide bonds, thereby coupling the functional groups Rx to the scaffold moiety. However, as opposed to (A), the scaffold is here encoded by the template, and therefore the scaffold is here part of the functional entity of a building block.

(C) Three building blocks are used. The nucleophilic amine (covalently attached to the template) attacks the ester carbonyl of the building block bound to coding region 3; the amine of the third monomer attacks the thioester of the next incorporated building block, and after incorporation of the third building block, the Horner-Wittig Emmans reagent of the building block reacts with the aldehyde of the third monomer under alkaline conditions. This forms the templated molecule. The double bond may be post-templating modified by hydrogenation to form a saturated bond, or alternatively, submitted to a Michael addition.

(D) The thiol of the scaffold reacts with the pyridine-disulfide of the incorporated building block. The amine of the scaffold reacts with the ester of the second incorporated building block. The double nitrile-activated alpha-position is acylated by the thioester of the next

building block in the presence of base. Finally, the aryliodide undergoes Suzuki coupling with the arylboronate of monomer 4 to yield the biaryl moiety.

(E) The incorporated building block acylates one of the primary amines. The aryliodide undergoes a Suzuki coupling by reaction with the next building block, and the benzylic amine is acylated by last incorporated building block.

(F) Acylation of the hydrazine followed by cyclization leads to formation of a hydroxypyrazole. After incorporation of the second building block, the arylbromide undergoes Suzuki coupling with the aryl boronate. Finally, the aldehyde reacts with the Horner-Wittig-Emmons reagent of the building block that is next incorporated, to yield an alpha, beta-unsaturated amide, which may be further modified or functionalized by either reduction with $H_2$/Pd-C or Michael addition with nucleophiles. Alternatively, a fourth building block might be used to template the coupling of a nucleophilic substituent at the double bond position.

Fig. 19 shows a general procedure of templated synthesis, wherein the reaction step may be performed under conditions where specific annealing of building blocks to the template is inefficient.

It may be desirable to perform the reaction step (or one of the other steps) under conditions where annealing of building blocks is in-efficient. To solve this potential problem, one may covalently link the incorporated building blocks, either chemically or by using a ligase (when the anti-codon comprises an oligonucleotide) or a polymerase (when the anti-codon is e.g. a nucleotide). In this set-up, the template is designed to fold back on itself.

In step 1, the two incorporated building blocks are incorporated and may be ligated together, and be linked to the template, during or after their incorporation. If desired, the conditions may now be changed to increase the efficiency of the reaction step that follows. Then, in step 2, the reactive groups X and Y are brought to react. Because the building blocks are covalently attached to each other (and to the template), the reaction can be performed under conditions where annealing of the building blocks to the template is inefficient. Reaction conditions that may not be compatible with efficient annealing and double helix structure include organic solvents, low salt and high temperature, all of which may be used with the set-up described in this figure.

After step 2 (reaction), the conditions are changed again, in order to allow efficient incorporation and covalent linkage of the next building block (step 3). This cycling between conditions that allow incorporation and ligation, and that allow reaction, is continued until the desired number of building blocks have been incorporated and reacted. Finally, some of the linkers are cleaved to give the templated molecule. As described above, the covalent coupling of the building blocks to each other allows the reaction between their reactive groups to be performed under more diverse conditions than would otherwise be possible. In addition, covalent coupling between building blocks makes it possible to use anti-codons comprising shorter recognition sequences. When the anti-codon comprises an oligonucleotide, it is generally preffered to use an oligonucleotide of at least fifteen nucleotides during incorporation, in order to obtain high efficiency of incorporation. However, if a ligase or chemical is used to covalently couple the building blocks, a shorter oligonucleotide (4-8 nucleotides) may be used. This will bring the reactive groups X and Y into closer proximity, and increase the local concentration of rective groups dramatically: If the distance between the reactive groups is decreased from 16 nucleotides to 4 nucleotides, this will increase the local concentration $4^3 = 64$. Everything else being equal, this will increase the rate of the reaction by 64-fold.

In order to change between conditions that allow incorporation and covalent coupling between building blocks, and conditions that allow the reaction to occur efficiently, the templates may be attached to a solid phase material (e.g., streptavidin beads if the templates are biotinylated), or the templates (with the building blocks associated to them) may be precipitated and resuspended in appropriate buffer during the steps of incorporation and reaction.

Fig. 20 shows various reaction types allowing simultaneous reaction and activation. Different classes of reactions are shown which mediate translocation of a functional group from one monomer building block to another, or to an anchorage point. The reactions have been grouped into three different classes: Nucleophilic substitutions, addition-elimination reactions, and transition metal catalyzed reactions These reactions are compatible with simultaneous reaction and activation.

(A) Reaction of nucleophiles with carbonyls. As a result of the nucleophilic substitution, the functional group R is translocated to the monomer building block initially carrying the nucleophile.
(B) Nucleophilic attack by the amine on the thioester leads to formation of an amide bond, in effect translocating the functional group R of the thioester to the other monomer building block.
(C) Reaction between hydrazine and β-ketoester leads to formation of pyrazolone, in effect translocating the R and R' functional groups to the other monomer building block.
(D) Reaction of hydroxylamine with β-ketoester leads to formation of the isoxazolone, thereby translocating the R and R' groups to the other monomer building block.
(E) Reaction of thiourea with β-ketoester leads to formation of the pyrimidine, thereby translocating the R and R' groups to the other monomer building block.

(F) Reaction of urea with malonate leads to formation of pyrimidine, thereby translocating the R group to the other monomer building block.

(G) Depending on whether Z = O or Z = NH, a Heck reaction followed by a nucleophilic substitution leads to formation of coumarin or quinolinon, thereby translocating the R and R' groups to the other monomer building block.

(H) Reaction of hydrazine and phthalimides leads to formation of phthalhydrazide, thereby translocating the R and R' groups to the other monomer building block.

(I) Reaction of amino acid esters leads to formation of diketopiperazine, thereby translocating the R group to the other monomer building block.

(J) Reaction of urea with α-substituted esters leads to formation of hydantoin, and translocation of the R and R' groups to the other monomer building block.

(K) Alkylation may be achieved by reaction of various nucleophiles with sulfonates. This translocates the functional groups R and R' to the other monomer building block.

(L) Reaction of a di-activated alkene containing an electron withdrawing and a leaving group, whereby the alkene is translocated to the nucleophile.

(M) Reaction of disulfide with mercaptane leads to formation of a disulfide, thereby translocating the R' group to the other monomer building block.

(N) Reaction of amino acid esters and amino ketones leads to formation of benzodiazepinone, thereby translocating the R group to the other monomer building block.

(O) Reaction of phosphonates with aldehydes or ketones leads to formation of substituted alkenes, thereby translocating the R" group to the other monomer building block.

(P) Reaction of boronates with aryls or heteroaryls results in transfer of an aryl group to the other monomer building block (to form a biaryl).

(Q) Reaction of arylsulfonates with boronates leads to transfer of the aryl group.

(R) Reaction of boronates with vinyls (or alkynes) results in transfer of an aryl group to the other monomer building block to form a vinylarene (or alkynylarene).

(S) Reaction between aliphatic boronates and arylhalides, whereby the alkyl group is translocated to yield an alkylarene.

(T) Transition metal catalysed alpha-alkylation through reaction between an enolether and an arylhallide, thereby translocating the aliphatic part.

(U) Condensations between e.g. enamines or enolethers with aldehydes leading to formation of alpha-hydroxy carbonyls or alpha,beta-unsaturated carbonyls. The reaction translocates the nucleophilic part.

(V) Alkylation of alkylhalides by e.g. enamines or enolethers. The reaction translocates the nucleophilic part.

(W) [2+4] cycloadditions, translocating the diene-part.

(X) [2+4] cycloadditions, translocating the ene-part.

(Y) [3+2] cycloadditions between azides and alkenes, leading to triazoles by translocation of the ene-part.

(Z) [3+2] cycloadditions between nitriloxides and alkenes, leading to isoxazoles by translocation of the ene-part.

Fig. 21 shows pairs of reactive groups (X) and (Y), and the resulting bond (XY).

A collection of reactive groups that may be used for templated synthesis as described herein are shown, along with the bonds formed upon their reaction. After reaction, cleavage may be required (e.g., see fig. 8).

Fig. 22 shows a method of increasing the proximity effect of the template: The Zipper-box.

Panel A discloses linkers carrying oligonucleotide zipper boxes (a) and (b) that are complementary. By operating at a temperature that allows transient interaction of (a) and (b), the reactive groups X and Y are brought into close proximity during multiple annealing and strand-melting events, which has the effect of keeping X and Y in close proximity in a larger fraction of the time than otherwise achievable. Alternatively, one may cycle the temperature between a low temperature (where the zipper boxes pairwise interact stably), and a higher temperature (where the zipper boxes are apart, but where the anti-codon remains stably attached to the codon of the template). By cycling between the high and low temperature several times, a given reactive group X is exposed to several reactive groups Y, and eventually will react to form an XY bond. As a final alternative, the temperature may be kept appropriately low that the two strands of the zipper-box (a and b) are stably associated. Independent on which of these protocols is followed, the building blocks must be added to the reaction mix at an appropriately high temperature where the interaction between the codon and anti-codon is specific. Once the building blocks have been specifically associated with the template, the temperature can be lowered, and the alternative protocols described above followed, in order to achieve a high reaction efficiency.

When the anti-codon is an oligonucleotide (e.g., DNA, RNA) or oligonucleotide analog (e.g., PNA, LNA), it may be practical to use a continuous nucleotide strand, comprising both the anti-codon, linker and zipper-box (see (B) below).

Panel B shows sequences of two DNA oligo-based building blocks. The anti-codon ("annealing region"), linker and zipper-box are indicated. Thus, in this example, one linear DNA molecule constitutes the anti-codon, the linker that connects the functional entity and the anti-codon, and the zipper-box. The reactive groups X (a carboxylic acid) and Z (an amine) are coupled to the 3'-end of DNA oligo 1 and the 5'-end of DNA oligo 2, respectively. A template sequence to which oligo 1 and oligo 2 would anneal might contain the follow-

ing sequence: 5'-CCGATGCAATCCAGAGGTCG-GCTGGATGCTCGACAGGTC.

Fig. 23 shows three methods of how the proximity effect can be increased: (A) Helix stacking, (B) Ligation and (C) Rigid linkers.
(A) Helix stacking. Two building blocks with oligonucleotide-based anti-codons anneal to their respective codons (in the figure, the left building block is a "scaffold" that carries four reactive groups, and the right building block carries a functional entity with e.g. one reactive group, i.e., the latter building block may carry the substituent that will become attached to the scaffold. Double helices tend to stack, especially if the sequence of the opposing ends of the helices has been designed so as to optimize this interaction (for example by the presence of the sequence GGG at the ends of the duplex structures). This stacking tendency will bring the two building blocks into closer proximity, in turn increasing reaction efficiency between the functional entities. If the "substituent-building blocks" have anti-codons with lower melting temperatures than that of the "scaffold-building block", the substituent building block may be removed after its reaction with the scaffold building block, before the next building block is incorporated. In this way, the template region between two reacting building blocks may be kept single stranded, allowing this region to loop out and let the two duplex structures stack during the reaction between the two building blocks.

(B) Ligation of building blocks. The anti-codons of two building blocks may be chemically or enzymatically ligated together. Coupling of two anti-codons will increase the annealing efficiency. Therefore, smaller anti-codons can be used if ligated together with the previously incorporated building block. As an example, first add a building block (or just an 20-nucleotide DNA oligo) with a melting temperature of e.g. 60 °C. Then add another building block (e.g., with a 8-nucleotide DNA anti-codon) with a low melting temperature and therefore only capable of transiently interacting with the template at the ambient temperature. If a DNA ligase is employed, or if the anti-codon can be

ligated to the anti-codon of the first building block chemically, then the second building block will become firmly attached to the template, despite its short length of just 8 nucleotides. Thus, ligation allows the use of shorter anti-codons, which in turn brings the reactive groups into closer proximity.

(C) Rigid linkers. By using linkers comprising one or more flexible regions ("hinges") and one or more rigid regions, the probability of two functional entities getting into reactive contact may be increased.

a. Symbol used for building block with a rigid part and two flexible hinges.

b. A building block with the characteristics described in (a). The building block contains a continuous oligonucleotide –strand, constituting both the anti-codon (horizontal line), and linker (vertical line) connecting the functional entity (FE) with the anti-codon. Annealing of a complementary sequence to the central part of the linker leads to formation of a rigid double helix; at either end of the linker a single-stranded region remains, which constitutes the two flexible hinges.

Fig. 24 discloses various cleavable linkers. A number of cleavable linkers are shown, as well as the agents that cleave them and the products of the cleavage-reaction. In addition, catalysts including enzymes and ribozymes, may also be used to cleave the linker. Exemplary enzymes are proteases (e.g. chymotrypsin), nucleases, esterases and other hydrolases.

Fig. 25 shows two different ways of templated synthesis by generating a new reactive group. In cases where the reaction of X and Y leads to formation of a new reactive group Z, this may be exploited to increase the diversity of the templated molecule, by incorporating building blocks carrying reactive groups Q that react with Z. Using this approach, the templated molecules may be very compact structures, and thus, this approach describes a method to make highly substituted (functionalized and diverse) libraries of molecules of relatively low molecular weight.

(A) First, a building block carrying a reactive group X and a building block carrying a reactive group Y is incorporated, whereafter X and Y react, leading to the formation of the Z bond. Then a building block carrying a reactive group Q is added, whereafter Z reacts with Q, to form the ZQ bond. In this example, both the reaction of X with Y, and of Z with Q, are reactions that involve simultaneous reaction and cleavage.

(B) First, a building block carrying a reactive group X and a building block carrying a reactive group Y is incorporated, whereafter X and Y react, leading to the formation of the Z bond. Then a building block carrying a reactive group Q is added, whereafter Z reacts with Q, to form the ZQ bond. In this example, the reaction of Z with Q does not involve simultaneous cleavage, wherefore an additional step of linker cleavage is introduced.

Fig. 25, example 1, shows a templated synthesis by generating a new reactive group. The reaction of the functional entities of the first three building blocks leads to formation of two double bonds, which may react with two hydroxylamines carried in by the building blocks added in the latter steps, and leads to formation of an ester, which may react with the hydroxylamine, encoded by a building block. Finally, the linkers are cleaved, generating the templated molecule.

Fig. 26 shows different methods of performing post-templating modifications on templated molecule. After the templating process has been performed, the templated molecules may be modified to introduce new characteristics. This list describes some of these post-templating modificiations.

Fig. 27 illustrates one preferred method for selection of template-displaying molecules.

Figure 28 shows the proposed complexes that may form when a reaction step is performed using set-ups that allow for stacking of DNA duplexes.

Figure 29 shows a autoradiography of a polyacrylamide gel analysis of the reaction between building blocks.

Figure 30 shows the Feuston 3 functional entity as well as the Feuston 5 ligand.

Figure 31 shows the structure of the pentenoyl protected aspartate.

Figure 32 shows the use of allylglycine building blocks.

Figure 33 shows the autoradiography of a polyacrylamide gel.

Figure 34 shows an Elisa analysis of the product of the two-step encoding process.

**Examples**

In the following examples, building blocks are used which contain a zipper box adjacent to the functional entity. The zipper box sequences are underlined below. The following buffers and protocols are used in the same three examples.

Buffers.
Buffer A (100 mM Hepes pH= 7,5; 1 M NaCl)
Buffer B (20 mM Hepes pH= 7,5; 200 mM NaCl)

5'-Labeling with $^{32}P$.
Mix 5 pmol oligonucleotide, 2 µl 10 x phosphorylation buffer (Promega cat#4103), 1 µl T4 Polynucleotide Kinase (Promega cat#4103), 1 µl $\gamma$-$^{32}P$ ATP, add $H_2O$ to 20 µl. Incubate at 37°C 10-30 minutes.

PAGE (polyacrylamide gel electrophoresis).

The samples are mixed with formamide dye 1:1 (98% formamide, 10 mM EDTA, pH 8, 0,025 % Xylene Cyanol, 0,025% Bromphenol Blue), incubated at 80°C for 2 minutes, and run on a denaturing 10% polyacrylamide gel. Develop gel using autoradiography (Kodak, BioMax film).

**Example 1**

**The effect of alternating temperature on reaction efficiency in the zipper box system.**

DNA-oligos:

X= Carboxy-dT (Glen Research, cat.no. 10-1035)

6= Amino-Modifier 5 (cat. Nr. 10-1905)

AH 316: 5'- 6GTAACAGACCTGTCGAGCATCCAGCT

AH 331: 5'-

CGACCTCTGGATTGCATCGGTGTTACX

AH140: 5'-

AGCTGGATGCTCGACAGGTCAGGTCGATCCGCGTTACCAG**TCTTGCC-TGAACGTAGTCGT**CCGATGCAATCCAGAGGTCG

Experimental.

Mix 10 µl Buffer A, 1 pmol AH 331 ($^{32}$P-labelled), 10 pmol AH 316, 5 pmol AH 140, and add $H_2O$ to 50 µl.

Anneal from 80° C to 30° C (-1° C / 30 sek). Then dilute 100 times in buffer B + 50 mM DMT-MM. (Prepared according to Kunishima *et al. Tetrahedron* (2001), **57**, 1551) dissolved in $ddH_2O$.

Incubate at one of 8 different temperature profiles o/n (6 different constant temperatures ( 15°C; 17,8°C; 22,7°C; 28,3°C; 31,0°C; or 35,0°C; or alternating between 10°C for 5 sec. and 35°C for 1 sec.); or alternating between

20°C for 5 sec. and 45°C for 1 sec). Analyze by 10% urea polyacrylamide gel electrophoresis.

Results.

The polyacrylamide gel analysis showed that a more efficient reaction results from alternating the temperature between 10 °C and 35 °C, rather than performing the reaction at a constant temperature of 15°C, 17,8°C, 22,7°C, 28,3°C, 31,0°C, or 35,0°C.

**Example 2**

**The effect of stacking on reaction efficiency.**

DNA-oligos:

X= Carboxy-dT (cat.no. 10-1035)

Z= Amino-Modifier C6 dT (cat.no. 10-1039)

6= Amino-Modifier 5 (cat.no. 10-1905)

AH36: 5'-
CGACCTCTGGATTGCATCGGTCATGGCTGACTGTCCGTCGAATGTGTC-
CAGTTACX

AH38: 5'- AGCTGGATGCTCGACAGGTCCCGATGCAATCCAGAGGTCG

AH51: 5'-
ZGTAACACCTGTGTAAGCTGCCTGTCAGTCGGTACTGACCTGTCGAG-
CATCCAGCT

AH137: 5'-ACGACTACGTTCAGGCAAGA

AH138: 5'-
TCTTGCCTGAACGTAGTCGTAGGTCGATCCGCGTTACCAGAGCTG-
GATGCTCGACAGGTCCCGATGCAATCCAGAGGTCG

AH139: 5'-CGACCTCTGGATTGCATCGG

AH143: 5'-
CTGGTAACGCGGATCGACCTTCATTTTTTTTTTTTTTTTTTTTTGGCT-
GACTGTCCGTCGAATGTGTCCAGTTACX
AH 202: 5'-TCTGGATTGCATCGGGTTACX
AH 270: 5'- 6GTAACGACCTGTCGAGCATCCAGCT
AH 286: 5'-
AGCTGGATGCTCGACAGGTCAAGTAACAGGTCGATCCGCGTTA-
TATCGTTTACGGCATTACCCGTATAGCCGCTAGATGCCCAACCATGACG
GCCCATAGCTTGCGGCTTGC
AH 320: 5'-
AGCTGGATGCTCGACAGGTCAGGTCGATCCGCGTTACCAGGCC-
CATAGCTTGCGGCTTGCTGCAGTCGATGGACCATGCCTCTTGCCT-
GAACGTAGTCGTCCGATGCAATCCAGAGGTCG
AH 321: 5'-CAAGAGGCAT
AH 322: 5'-TCAGGCAAGAGGCATGGTCC
AH 342: 5'-TACTTGACCTGTCGAGCATCGTTACX
AH 343: 5'- 6GTAACCAGCTGCAAGCCGCAAGCTATGGGC

**Experimental.**

Mix buffer A and relevant oligos (see table below).

| Experiment | Oligo 1 ($^{32}$P-labelled) | Oligo 2 | Oligo 3 Template | Oligo 4 | Oligo 5 | Buffer A | $H_2O$ to |
|---|---|---|---|---|---|---|---|
| 1 | 5 pmol AH 36 | 10 pmol AH 51 | 10 pmol AH 38 | | | 2 µl | 10 µl |
| 2 | 5 pmol AH 143 | 10 pmol AH 51 | 10pmol AH 138 | 10 pmol AH 139 | 10 pmol AH 137 | 2 µl | 10 µl |
| 3 | 1 pmol AH 202 | 10 pmol AH 270 | 5 pmol AH 320 | | | 10 µl | 50 µl |
| 4 | 1 pmol AH 36 | 10 pmol AH 51 | 5 pmol AH 320 | | | 10µl | 50 µl |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5 | 1 pmol AH 202 | 10 pmol AH 270 | 5 pmol AH 320 | 50 pmol AH 321 | | 10 µl | 50 µl |
| 6 | 1 pmol AH 36 | 10 pmol AH 51 | 5 pmol AH 320 | 50 pmol AH 321 | | 10µl | 50 µl |
| 7 | 1 pmol AH 202 | 10 pmol AH 270 | 5 pmol AH 320 | 50 pmol AH 322 | | 10 µl | 50 µl |
| 8 | 1 pmol AH 36 | 10 pmol AH 51 | 5 pmol AH 320 | 50 pmol AH 322 | | 10µl | 50 µl |
| 9 | 0,2 pmol AH 342 | 2 pmol AH 343 | 1 pmol AH 286 | | | 2 µl | 10 µl |
| 10 | 0,2 pmol AH 342 | 2 pmol AH 343 | 1 pmol AH 286 | 4 pmol AH 356 | | 2 µl | 10 µl |
| 11 | 0,2 pmol AH 342 | 2 pmol AH 343 | 1 pmol AH 286 | 4 pmol AH 357 | 4 pmol AH 358 | 2 µl | 10 µl |

Anneal from 80°C to 30°C (-1°C/min). Add 0,5 M DMT-MM. (Prepared according to Kunishima *et al. Tetrahedron* (2001), 57, 1551 ) dissolved in $H_2O$. to a final concentration of 50 mM. Incubate at 10°C for 5 sec. and then 25°C for 1 sec. Repeat o/n.

Analyze by 10% urea polyacrylamide gel electrophoresis.

**Results.**

In order to test the effect of stacking of DNA duplexes on reaction efficiency, we designed a number of different set-ups of templates and building blocks (see Figure 28). The following conclusions were reached:

Figure 28,1 and Figure 29, lane 1: Reference reaction between two building blocks annealed to adjacent sites on the template. As expected an efficient reaction is observed. In this set-up, the two building blocks anneal to the template and thereby form DNA duplexes that can stack onto each other.

Figure 28,2 and Figure 29, lane 2: In this set-up, the two building blocks anneal to adjacent sites on the template. However, the two DNA-duplexes stack onto each other, basically forming one long DNA duplex. This rigid duplex does not allow the two building blocks to bend around the flexible hinge that might otherwise be present at the connection point between the two duplexes (i.e. the position of the nick in the DNA). Consequently, no significant reaction between the two building blocks is observed.

Figure 28,3 and Figure 29, lane 3; and Figure 28,4 and Figure 29, lane 4: Despite the fact that the two building blocks anneal to sites separated by 80 nucleotides, the reaction is still very efficient. We speculate that this is because of stacking, i.e. the intervening 80 nucleotides are looped out as a consequence of this, and therefore, the two functional entities are brought into close proximity.

In the experiment of Figure 29, lane 3 the linker that connects the functional entity to the complementing element is short (5 nucleotides); in Figure 29, lane 4 it is long (35 nucleotides). However, both linker lengths result in an efficient reaction.

Figure 28, 5 and Figure 29, lane 5; and Figure 28, 6 and Figure 29, lane 6: The annealing sites and separation between them are identical to those of the experiment described above (Figure 28, 3 and 4; Figure 29, lanes 3 and 4). In addition, a short oligo (10 nucleotides) has been annealed to the central region of the template. This result in a drastic decrease in reaction efficiency for the building blocks with the short linkers (lane 5); the reaction efficiency of the building blocks with the long linkers is only slightly affected if at all by the annealing of the short oligo. As indicated by the suggested structure of the complexes (Figure 28, 5 and 6), we believe this is because of stacking of the 3 DNA duplexes to generate an "extended" duplex: The short linkers cannot reach across the extended duplex; the long linkers can reach

across the extended duplex structure and the reaction efficiency is not significantly affected.

Figure 28, 7 and Figure 29, lane 7; and Figure 28, 8 and Figure 29, lane 8: As immediately above, except that a 20 nucleotide long oligo is annealed to the central region of the template. In this case none of the linkers (short or long) can reach across the extended duplexes, and as a result no or little reaction is observed.

Figure 28,9 and Figure 29, lane 9; Figure 28,10 and Figure 29, lane 10; and Figure 28,11 and Figure 29, lane 11: In these experiments the building blocks are oriented the "other way", i.e. the linker connecting the complementing element and the functional entity is near the ends of the template. Additionally, the complementing element of the left building block contains a 5-nucleotide sequence that is complementary to other right end of the template. As a result, the building block should be capable of circularizing the template, as depicted in Figures 27, 9-11. These circular structures should also be stabilized by an extended duplex structure across the ends of the template. In the experiments of lanes 10 and 11, a short oligo (10 nucleotides) or two longer oligos (each 20 nucleotides) are annealed to the central region. This has no effect on the reaction efficiency, in correlation with the proposal that the building blocks stack onto each other through a circularization of the template, thereby bringing the functional entities into close proximity.

**Example 3**

**Single step transfers of functional entities.**

DNA-Oligos:

7=Thiol-Modifier C6 S-S (Glen Research, cat.no.10-1936)

Z=Amino-Modifier C6 dT (10-1039)

P= PC Spacer (10-4913)

AH136: 5'- AGCTGGATGCTCGACAGGTCTCTTGCCTGAACGTAGTCG-TCCGATGCAATCCAGAGGTCG

AH 174: 5'-TACGTTCAGGCAAGAGT6CCAGTTAC7

AH 190: 5'- ZGTAACACCTGPTGACCTGTCGAGCATC

**Experimental:**

Loading of NHM on the DNA-oligo:

Dry 10 nmol DNA oligo (AH174) and then resuspended in 50 µl 100 mM DTT (1,4-Dithio-L-Threitol D-9760 Sigma) in 50 mM Phosphate buffer pH=8. Incubate at 37°C for 1 hour.

Purification on Microspin G-25 (Amersham Biosciences, 27-5325-01).

Add 50 µl 200 mM NHM ( N-Hydroxymaleimide Fluka 55510) and incubate at 25°C for 2 hours.

Purification on Microspin G-25 equilibrated in $H_2O$.

Loading of building blocks (4-pentenoic-acid, ß-ala-Boc or $CH_3COOH$) on the NHM-DNA-oligo:

Mix 50 µl 100 mM EDC and 50 µl 100 mM building block. Incubate at 25°C for 30 minutes.

Then mix 500 pmol NHM-DNA-oligo (AH174-NHM) and 10 µl of the EDC/ building block mix from above. Add 100 mM MES pH=6 to 20 µl. Incubate at 25°C for 5 minutes.

Purification on Micro Bio-Spin Chromatography Columns P6 (Bio-Rad 732-6221) equilibrated in 100 mM MES pH=6.

Transfers:

Mix 350 pmol AH136, 300 pmol AH190 and 500 pmol building block loaded AH174. Add Buffer A to 50 µl.

Anneal from 60°C to 25°C (-1°C/ 30 sec.)

Incubate at 10°C for 5 sec. and then 25°C for 1 sec. Repeat o/n.

Purification on Micro Bio-Spin Chromatography Columns P6 equilibrated in $H_2O$.

**Results:**

The transfers were analyzed by MS, see table below. Transfer efficiencies of 20-34% were observed.

| Transfer efficiency | | |
|---|---|---|
| 4-pentenoic-acid | ß-ala-Boc | $CH_3COOH$ |
| 33-34% | 20-23% | 29-33% |

**Example 4**

**Multistep transfer of functional entities to a scaffold oligonucleotide**

In this example three functional entities are transferred to an amino modified scaffold oligo by a three step reaction, and analyzed by a denaturing acrylamide gel using radio labelling.

Loading of functional entities on modified oligonucleotides to create building blocks.

5 nmoles of three carboxylic acid modified building block oligos [AH 155; 5'CTG GTA ACG CGG ATC GAC CTG TTA CT-COOH 3'; AH 272 5'ACG ACT ACG TTC AGG CAA GAG TTA CT-COOH 3' and AH 202 5'-TCT GGA TTG CAT CGG CTG TTA CT-COOH 3'] (all oligonucleotides described ordered from DNA technology, Aarhus, Denmark) one from each of the three positions corresponding to the template were loaded with β-Alanine methyl ester coupled to allylglycine n-Boc followed by Boc deprotection (β-AlaOMe AG). The loading was done by incubating each of the oligos with 10 mM β-

AlaOMe AG, 75 mM DMT-MM in 150 mM Hepes-OH buffer, pH 7,5 to a final volume of 50 µl at 25°C shaking overnight. Then adding 5 µl 1 M $NH_4$-acetate, incubated at 25°C for 10 min, then spin column purified with $ddH_2O$ equilibrated columns (Micro Bio-Spin chromatography columns P-6, Bio-Rad). The deprotection of the methyl group protected acid was done by adding 0,5 µl 2M NaOH to the oligos and incubating for 10 min at 80°C. Lastly the oligos were spin column purified and loadings confirmed by mass spectrophotometry.

Transfers of functional entities to scaffold oligo.

In order to be able to analyze the functional entity transfers using acrylamide gel analysis, the scaffold oligo [MDL251 5'amino-C6 dT-ACC TGT CGA GCA TCC AGC T 3'] was radioactively labelled in the 3' end. 50 pmol of the oligo was labelled with 10 µl ddATP αP32 (Amersham Biosciences) by adding 4 µl 10X NEbuffer 4, 4µl 10X CoCl2 and 35 units of terminal deoxynucleotide transferase (New England Biolabs) and water to a final volume of 40 µl. Mixture incubated at 37°C for 1 hour. Labeled oligo purified using ddH2O equilibrated spin column.

12,5 pmol of the labeled scaffold oligo, 125 pmol loaded building block oligo AH 202, corresponding to position three on the template and 62,5 pmol template [AH 154 5' AGC TGG ATG CTC GAC AGG TCA AGT AAC AGG TCG ATC CGC GTT ACC AGT CTT GCC TGA ACG TAG TCG TCC GAT GCA ATC CAG AGG TCG 3'] was incubated in a final volume of 45 µl containing 20 mM Hepes-OH pH 7,5, 200 mM NaCl buffer. The oligos were annealed by heating to 80°C and slowely going down to 20°C (1°/min) using a thermocycler (Eppendorf, Mastergradient) Following the annealing 5 µl 0,5M DMT-MM was added. Sample crosslinked, see figure 32 overnight cycling at 10°C 10 sec/35°C 1 sec.

The sample was spin column purified and the crosslinked product cleaved to give first transfer of β-Ala to scaffold oligo amine by adding 10 µl 25 mM I2 dissolved in 1:1 tetrahydrofuran:H2O and incubated at 37°C for 1,5 hours. Followed by addition of 1,5 µl 1 M β-mercapotethanol and then purified with two equilibrated spin columns. The sample was completely dried down and oligos redissolved in 30 µl ddH2O. Transfer 2, oligo AH 272 and transfer 3, AH 202 were done in the exact same way as just described including the annealing, crosslinking and cleavage. For each remaining round adding same amount of building block oligo, 125 pmol.

Samples for analysis were taking out along the way, before and after crosslinking for the three transfers, which were analyzed on a 10% acrylamide denaturing gel, see fig 33. As can be seen, crosslinking efficiency (step 1) was approximately 50% (Figure 33, lane 1). This was followed by an almost 100% efficient cleavage (lane 2), which results in the transfer of the β - Ala moiety onto the scaffold. This is followed by the crosslinking/cleavage of step 2 and 3 (lanes 3+4, 5+6) to generate the final product on the scaffold oligo. The product thus contains the three transferred β-Ala moieties.

**Example 5**

Two-step transfer and functional analysis by ELISA.

In this example two entities are transferred to a scaffold oligo by a two-step reaction to produce a ligand, Feuston 5 (see Figure 30) that binds to the αVβ3 integrin receptor. The product of the two-step process was analyzed by Elisa.

Loading of functional entities on modified oligonucleotides to create building blocks.

Two building block oligos were used, AH 155 (see above) loaded with Feuston 3 allylglycine. Feuston 3 is a derivative of the Feuston 5 ligand see fig 30 (F3OMeAG) and AH 272 (see above) loaded with glycine allylglycine (GlyO-

MeAG) according to the above protocol (example Xa) for loadings of allylglycine functional entities to carboxylic acid modified oligos. 10 nmoles of each was loaded in two reactions each.

To create the Feuston 5 ligand aspartate is also needed. Therefore aspartate which was loaded as a pentenoyl (amine) and methyl (carboxylic acid) protected functional entity see Figure 31, to an amino modified scaffold oligo [AH 270 ;5' amino-GTA ACG ACC TGT CGA GCA TCC AGC T 3']. The loading was done by mixing 25 µl 150 mM EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Fluka), 25 µl NHS (N-hydroxysuccinimide, Sigma) and 5 µl 100 mM of the pentenoyl protected aspartate functional entity, all reagents were dissolved in N,N-dimethylformamide, DMF. Incubated at 25°C for 40 min. To this mixture 5 nmol of the scaffold oligo, AH 270 resuspended in 30 µl 150 mM Hepes-OH pH 7,5 was added and this incubated shaking over night at 25°C. The amine pentenoyl protection group was deprotected by adding 20 µl 25 mM I2 dissolved in 1:1 tetrahydrofuran: water and incubated at 37°C for 2 hours. Followed by spin column purification, and loading confirmed by mass spectrum analysis.

Transfers of functional entities to scaffold oligo.

The transfers were done in the same manner as described above, but using larger amounts of oligo to ensure there being enough ligand created to give a sufficient signal in the ELISA. For the first round the following amounts were used: 850 pmol loaded scaffold oligo; AH 270, 7500 pmol loaded building block oligo; AH 272 and 3250 pmol template oligo AH 140 [ 5' AGC TGG ATG CTC GAC AGG TCA GGT CGA TCC GCG TTA CCA GTC TTG CCT GAA CGT AGT CGT CCG ATG CAA TCC AGA GGT CG 3']. The second round, adding 7500 pmol loaded building block oligo AH 155 for a transfer. The created Feuston 5 ligand on the scaffold oligo still had a methyl group protected acid on the aspartate, which was deprotected just as described before. By adding 0,5 µl 2 M NaOH to the oligos and incubating at 80°C for

10 min. The sample this time though was pH calibrated with 0,5 µl 2 M HCl and was now ready for the ELISA analysis.

ELISA assay

Maxisorb plates (Nunc Immunomodule U8 Maxisorp. Biotecline) were coated with αVβ3 integrin receptor 0,1 µg/well in PBS over night at 4°C. The wells were blocked with 300 µl blocking buffer containing PBS, 0.05% Tween 20 (Sigma), 1% BSA (Sigma), 0.1 mg/mL herring sperm DNA (Sigma), for 3 hours at room temperature. Wells were washed 5 * 300 µl using wash buffer containing PBS, 0.05% Tween 20, 1% BSA. The sample prepared above containing the displayed Feuston 5 ligand on a scaffold oligo was added to a well, control for the experiment being a 20 mer oligo loaded with the RGD peptide, a well known and well described ligand for this integrin receptor (loaded according to above described method for the pentenoyl and methyl protected aspartate functional entity). The incubation with these ligands was done in ligand binding buffer containing PBS, 1 mM MnCl2, 1 mg/mL BSA at room temperature for one hour. Washed in washing buffer 5 * 300 µl. Incubated with 100 µl horseradish peroxidase-streptavidine (Endogen) diluted 1:10000 times in wash buffer, incubated for one hour at room temperature. Washed again in 5 * 300 µl wash buffer. 100 µl 3, 3', 5,5'-tetrametylbenzidine hydrogenperoxidase (TMB substrate, Kem-en-tec) added and incubated at room temperature until color development. 100 µl 0,2 M sulphuric acid added, color measured at 450 nm, see figure 34. As can be seen the Feuston 5 ligand generated by the two-step encoding procedure is active and binds the integrin receptor with relatively high efficiency.

**Claims**

1. A method for the manufacture of a library of complexes comprising templated molecules, said method comprises the steps of

a) providing a plurality of different templates comprising a number of coding regions and a reactive group, wherein each coding region of a specific template specifies a unique codon,

b) providing a plurality of different building blocks, each building block comprising an anti-codon, a functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon of each building block complements a unique codon of a template, and the functional entity comprises at least one reactive group,

c) contacting the plurality of different templates with a subset of the plurality of different building blocks, said subset having anti-codons which complement the unique codons of a specific coding region, said contacting being performed under conditions which allow specific hybridisation of the anti-codons to the unique codons of the templates,

d) reacting the reactive group of the template and the reactive group of the building block to obtaining a chemical connection,

e) contacting under conditions allowing specific hybridisation, the plurality of different templates harbouring the nascent templated molecules with a further subset of the plurality of building blocks, said subset having anti-codons complementary to the unique codons of a coding region in the vicinity of the coding region harbouring the nascent templated molecules,

f) allowing the functional entities of the subset of further building blocks to form a chemical connection to the nascent templated molecules,

g) optionally, cleaving one or more of the linkers, provided that at least one linker remains to connect the nascent templated molecule with the template which directed the synthesis thereof,

h) optionally repeating steps e) through g),

i) obtaining a templated molecule attached via the linker one or more building blocks to the template which directed the synthesis thereof.

2. The method according to claim 1, wherein the reactive group of step a) comprised by the template is covalently attached to the template.

3. The method according to claim 1, wherein the reactive group of the template is non-covalently attached to the template.

4. The method according to claim 3, wherein the reactive group of the template is covalently attached to a complementing element hybridised to the template.

5. The method according to claim 3 or 4, wherein the reactive group of the template is part of a building block.

6. The method according to any of the claims 3 to 5, wherein the building blocks harbouring the reactive group of step a) and the subset of building blocks contacted with the templates in step c) are positioned next to each other.

7. The method according to any of the preceding claims, wherein the individual coding regions of the plurality of templates are positioned next to each other in a linear sequence.

8. The method according to claims 1 to 6, wherein the template is branched.

9. The method according to any of the claims 1 to 8, wherein coding regions are separated by a spacer group.

10. The method according to claim 9, wherein the spacer group identifies the neighbouring coding region or unique codon.

11. The method according to any of the preceding claims, wherein the number of coding regions is 3 to 100.

12. The method according to any of the preceding claims, wherein the number of unique codons within a coding region is between 1 and 10,000.

13. The method according to any of the preceding claims, wherein each unique codon is a sequence of 3 to 100 nucleic acid monomers.

14. The method according to claim 13, wherein each unique codon comprises a sequence of 8 to 30 nucleic acid monomers.

15. The method according to any of the preceding claims, wherein the individual unique codon:anti-codon hybrids within a specific coding region have a similar annealing temperature.

16. The method according to any of the claims 1 to 14, wherein the individual unique codon:anti-codon hybrids within a specific coding region have a different annealing temperature.

17. The method according to any of the preceding claims, wherein the functional entity of a building block comprises a reactive group capable of forming a connection to a reaction partner of another functional entity or nascent templated molecule.

18. The method according to any of the preceding claims, wherein the functional entity of a building block comprises a reactive group capable of forming a connection to a reactive group of another functional entity or nascent templated molecule through a bridging fill-in group.

19. The method according to any of the preceding claims, wherein the linker is attached to the anti-codon oligonucleotide at a central area thereof.

20. The method according to any of the preceding claims, wherein the anti-codon and the linker is a contiguous linear oligonucleotide.

21. The method according to any of the preceding claims, wherein the linker is attached to the functional entity through a reactive group capable of forming a connection to another functional entity or a nascent templated molecule.

22. The method according to claim 21, wherein the linker is capable of being cleaved simultaneously with the formation of the connection.

23. The method according to any of the preceding claims, wherein the reactive groups involved in the formation of the connection between functional entities or a functional entity and a nascent templated molecule are reactions partners.

24. The method according to any of the preceding claims, wherein the subset in steps c) comprises building blocks having anti-codons which form hybrids with unique codons in a coding region neighbouring the reactive group of the template.

25. The method according to any of the preceding claims, wherein the subset in step e) comprises building blocks having anti-codons which form hybrids with unique codons in a coding region neighbouring the building block harbouring the nascent templated compound.

26. The method according to claims 24 or 25, wherein the subset is formed by adding the building blocks separately.

27. The method according to any of the claims 24 or 25, wherein the subsets in steps c) or e) are formed by directing the annealing temperature of the individual building blocks.

28. The method according to any of the preceding claims, wherein the anticodon of a building block with a functional entity is ligated to the anti-codon of a building block harbouring a nascent molecule prior to establishing the connection between the functional entity and the nascent molecule being prepared.

29. The method according to any of the preceding claims, wherein building blocks intended to interact with each other each are provided with a part of a molecule pair being capable of reversible interaction.

30. The method according to claim 29, wherein the one part of the molecule pair is present on the linker, close to the functional entity or nascent templated molecule.

31. The method according to claims 29 or 30, wherein the one part of the reversible interacting molecule pair of a first building block is an oligonucleotide and the other part of the reversible interacting molecule pair of a second building block intended to interact with the first building block is a complementing oligonucleotide.

32. The method according to the preceding claims 29 to 31, wherein the annealing temperature of an interacting molecule pair is lower than the annealing temperatures for the unique codon:anti-codon hybrids of the involved building blocks.

33. The method of claim 32, wherein the annealing temperature of the reversible interacting molecule pair is below room temperature but above 5°C.

34. The method according to claim 33, wherein the annealing temperature is between 10°C and 20°C.

35. The method according to any of the preceding claims, wherein the linker is rigid and attached the anti-codon through a molecular hinge.

36. The method according to claim 35, wherein the rigid linker is a double stranded oligonucleotide.

37. The method according to claim 35 or 36, wherein the molecular hinge is a single stranded region of the building block.

38. The method for the manufacture of a library according to claim 1, wherein the complexes obtained comprise templated molecules attached to the template which templated the syntheses thereof via a single building block.

39. The method according to claim 1, comprising the further step of connecting the templated molecule with the template which directed the syntheses thereof, or a complementing template, via a covalent link.

40. The method according to claim 39, wherein the covalent link is selectively cleavable to provide for a release of the templated molecule.

41. The method according to claim 1, wherein the templated molecules of the library complex are polymers.

42. The method according to any of the claims 1 to 41, wherein the optional cleavage of some or all of the linkers of step g) are not performed.

43. The method according to claim 42, comprising the further step of cleaving all but one linker after the formation of the templated molecule.

44. The method according to any of the preceding claims, wherein the anti-codons following the cleavage of the linker attached thereto, remain hybridised to the unique codons.

45. The method according to claim 44, wherein the anti-codons attached to the templates are ligated together to create a complementary template.

46. The method according to claim 1, comprising the further step of transferring the templated molecule to an anchorage point on the template, or a sequence complementing the template, to establish an effective chemical connection.

47. The method according to claim 46, wherein the complementing sequence has a higher annealing temperature than one or more of the building blocks.

48. The method according to claim 1, comprising the further step of connecting the templated molecule with a complementary template via a covalent link.

49. The method according to claim 48, wherein the template is covalently connected to the complementing template.

50. The method according to claim 48, wherein the covalent link is selectively cleavable to provide for a separation of the templated molecule from the complementary template.

51. A library of complexes obtainable according to any of the claims 1 to 45.

52. A method of enriching a library of complexes comprising templated molecules with respect to a predetermined activity, said enrichment method comprising the steps of:

i) establishing a first library of complexes comprising templated molecules, said library being obtainable according to any of the claims 1 to 50,

ii) exposing the library to conditions enriching the library with complexes having the predetermined activity,

iii) amplifying the complexes of the enriched library,

iv) optionally, repeating step ii) to iii), and

v) obtaining an enriched library having a higher ratio of complexes comprising templated molecules with the predetermined activity.

53. The method of claim 52, wherein step iii) comprises a $10^1$ to $10^{15}$-fold amplification.

54. The method of claim 52, wherein the steps ii) and iii) are repeated at least 2, 3, 5 times, such as at least 10 times, such as at least 15 times.

55. The method of claim 52, further comprising a step of identification of the complexes having the predetermined activity.

56. The method of claim 52, wherein the identification is conducted by analysing the template and/or complementary template associated with the molecule.

57. The method of claim 52, wherein the conditions enriching the library comprises contacting a binding partner to the templated molecules of interest.

58. The method according to claim 57, wherein the binding partner being directly or indirectly immobilised on a support.

59. The method according to claim 52, wherein the enrichment is conducted by screening for complexes having an affinity for – or an effect on – a target molecule or a target entity.

60. The method according to claim 52, wherein the enrichment is conducted by selection for catalytic activity.

61. The method of claim 52, wherein the conditions enriching the library involves any one or more of electrophoretic separation, gelfiltration, immunoprecipitation, isoelectric focusing, centrifugation, and immobilization.

62. The method of claim 52, wherein the conditions enriching the library comprises providing cells capable of internalising the templated molecule, or performing an interaction with the templated molecule having the desired predetermined activity.

63. The method according to claim 52, wherein the amplification of the complexes of the enriched library comprises the steps of

A. contacting the library of complexes with amplification means,
B. amplifying the templates or the complementing templates, and
C. conducting the method according to any of the claims 1 to 50 using the amplification product of step B as templates.

64. A method for the manufacture of a complex of a templated molecule attached to the template which directed the synthesis thereof, said method comprises the steps of

a) providing a template comprising a number of coding regions and a reactive group, wherein each coding region specifies a unique codon,
b) providing a plurality of different building blocks, each building block comprising an anti-codon, a functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon of

each building block complements a unique codon of the template, and the functional entity comprises at least one reactive group,

c) contacting the template with a building block having an anti-codon which complements the unique codon of a specific coding region, said contacting being performed under conditions which allow specific hybridisation of the anti-codon to the unique codon of the templates,

d) reacting the reactive group of the template and the reactive group of the building block to obtaining a chemical connection,

e) contacting under conditions allowing specific hybridisation, the template harbouring the nascent templated molecule with a further building block having an anti-codon complementary to the unique codon of a coding region in the vicinity of the coding region harbouring the nascent templated molecule,

f) allowing the functional entity of the further building block to form a chemical connection to the nascent templated molecule,

g) optionally, cleaving one or more of the linkers, provided that at least one linker remains to connect the nascent templated molecule with the template which directed the synthesis thereof,

h) optionally repeating steps e) through g),

i) obtaining a templated molecule attached via the linker of one or more building blocks to the template which directed the synthesis thereof.

60. A method for preparing a templated molecule, comprising the further step of claim 59 of cleaving the linker(s) of the one or more building blocks to release the templated molecule.

Fig. 1

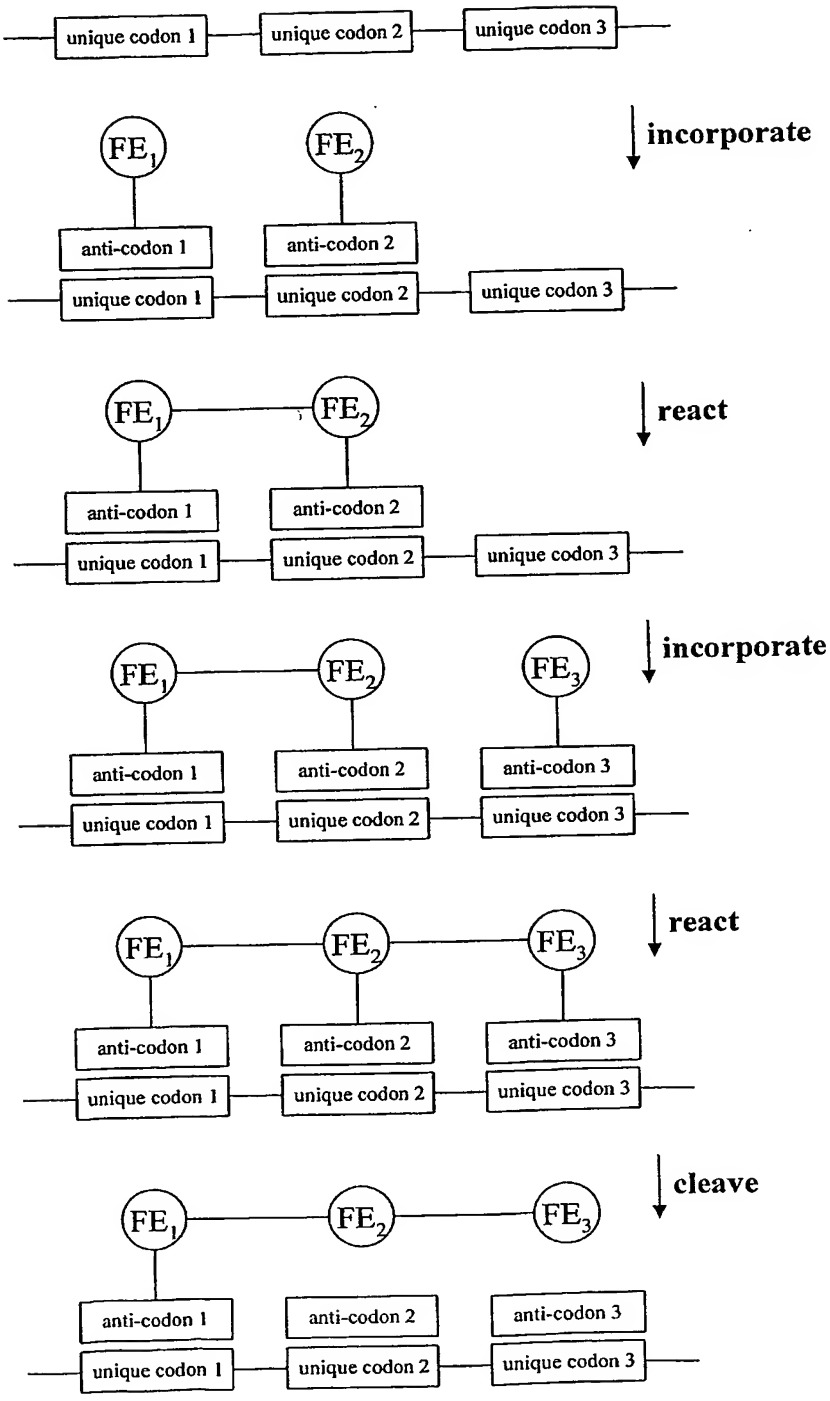

unique codon 1
unique codon 2
unique codon 3
incorporate
FE1
FE2
anti-codon 1
anti-codon 2
unique codon 1
unique codon 2
unique codon 3
react
FE1
FE2
anti-codon 1
anti-codon 2
unique codon 1
unique codon 2
unique codon 3
incorporate
FE1
FE2
FE3
anti-codon 1
anti-codon 2
anti-codon 3
unique codon 1
unique codon 2
unique codon 3
react
FE1
FE2
FE3
anti-codon 1
anti-codon 2
anti-codon 3
unique codon 1
unique codon 2
unique codon 3
cleave
FE1
FE2
FE3
anti-codon 1
anti-codon 2
anti-codon 3
unique codon 1
unique codon 2
unique codon 3

Fig. 2

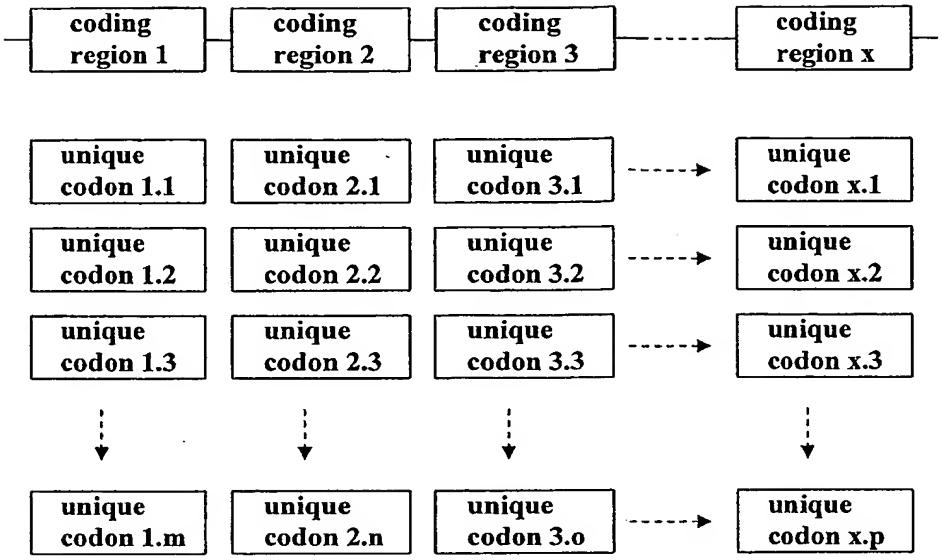
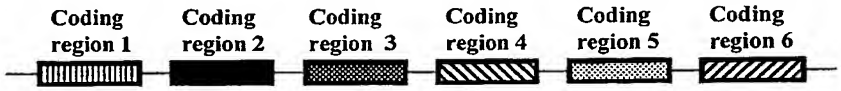

coding region 1
coding region 2
coding region 3
coding region x
unique codon 1.1
unique codon 2.1
unique codon 3.1
unique codon x.1
unique codon 1.2
unique codon 2.2
unique codon 3.2
unique codon x.2
unique codon 1.3
unique codon 2.3
unique codon 3.3
unique codon x.3
unique codon 1.m
unique codon 2.n
unique codon 3.o
unique codon x.p

**Fig. 3. An oligonucleotide-based building block. Example of coding region design, allowing for high building block diversity.**

**A.**




| Coding region | sequence | number of unique codons |
|---|---|---|
| 1 | XXXXXATATTTXXXXX | 1024 |
| 2 | XXXATTTTAXXXXXXX | 1024 |
| 3 | XTAATTTXXXXXXXXX | 1024 |
| 4 | XXATXXATXXATXXXX | 1024 |
| 5 | GCCCGATTAAAXXCCG | 4 |
| 6 | XAXAXTTXTTXXXGGG | 128 |

X = G or C

**B.**

Codon 1 GCGCGATATTTGGGCC
Anti-codon 1 CGCGCTATAAACCCGG

Codon 6 GAGAGTTCTTCGCGGG
Anti-codon 6 CTCTCAAGAAGCGCCC

**Fig. 4. Building blocks.**

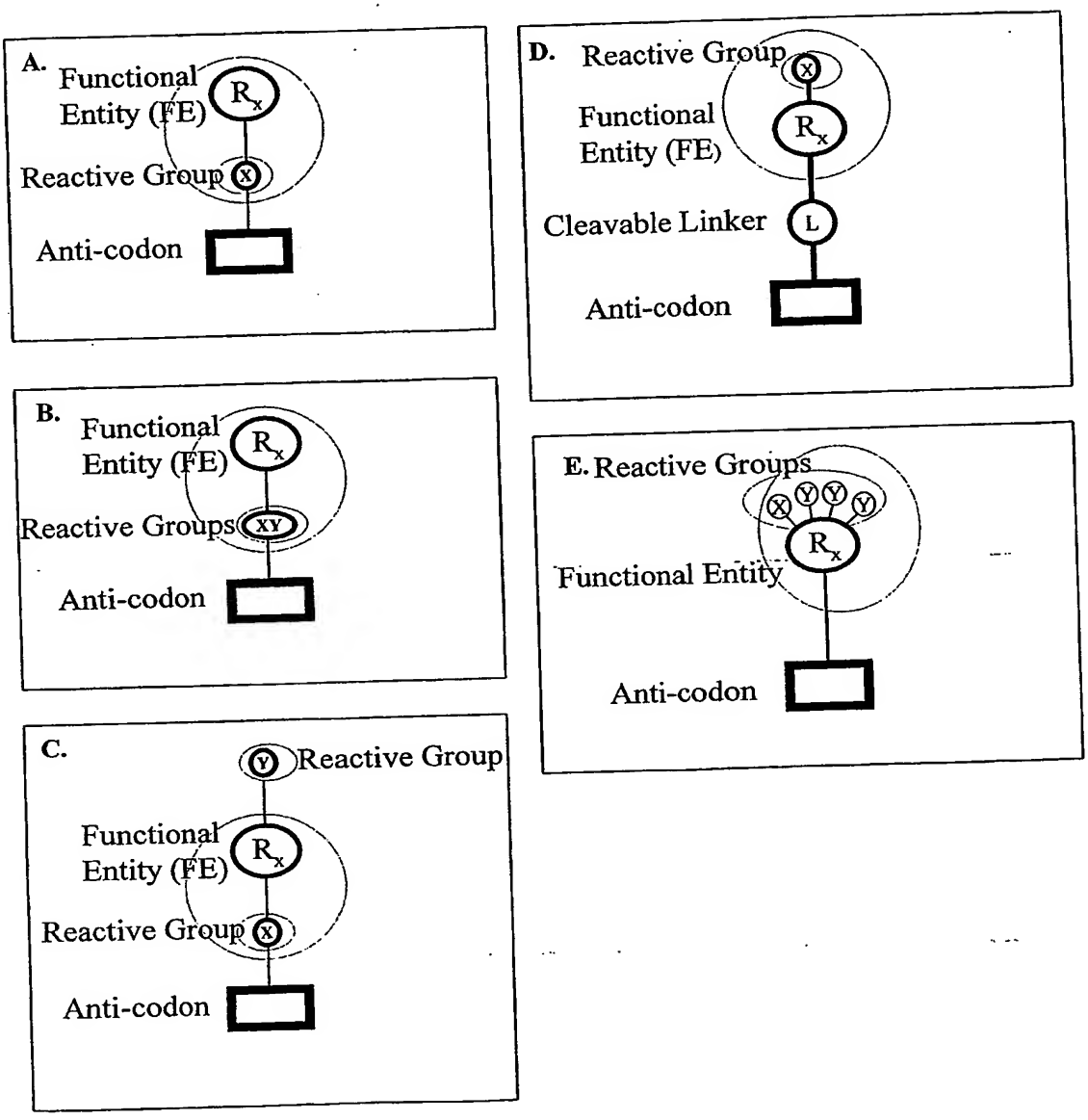


**Fig. 5. Exemplary monomer Building Blocks.**

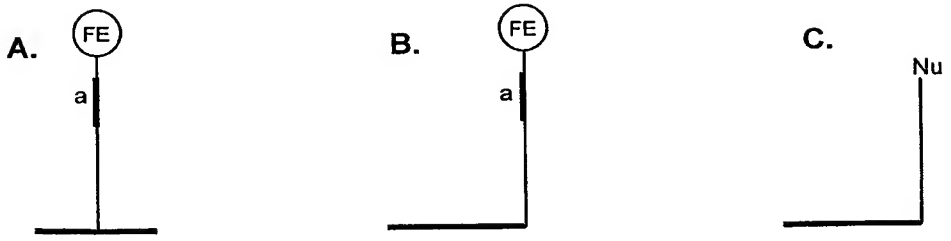


**Fig. 6. Preparation of Building Blocks. General examples**

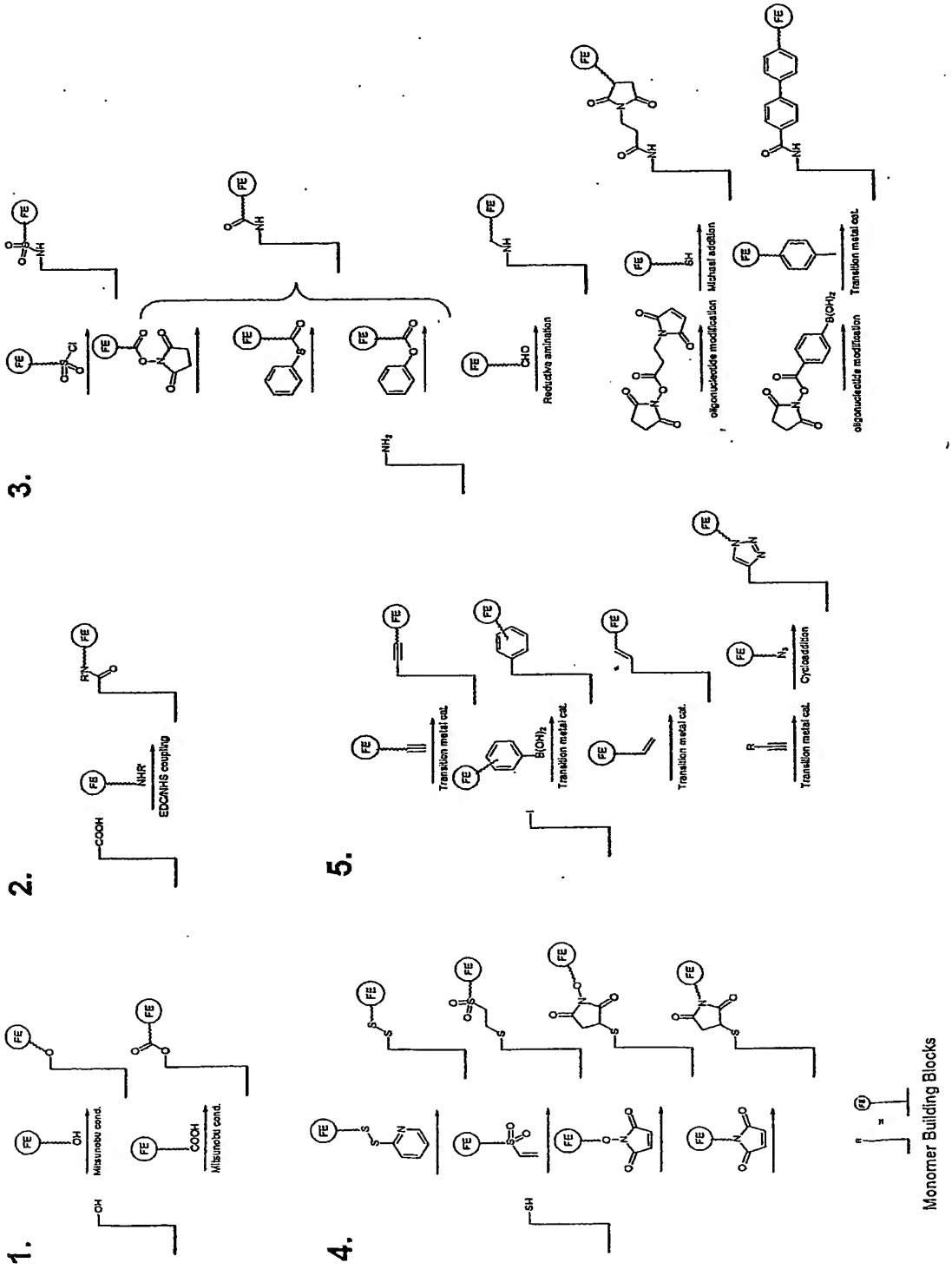


**Fig. 7. Design and synthesis of specific building blocks**

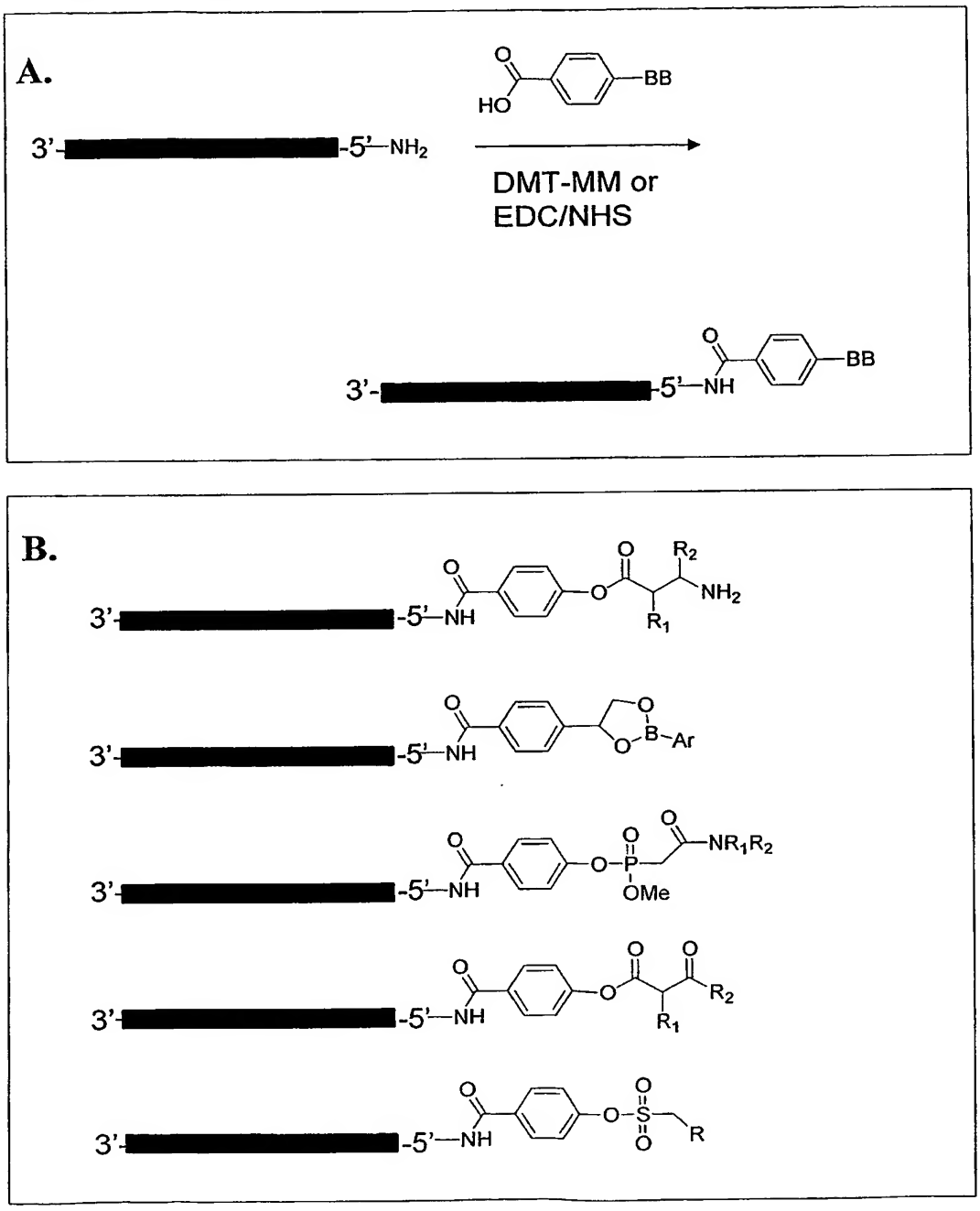


Fig. 8. Templated synthesis of a polymer.

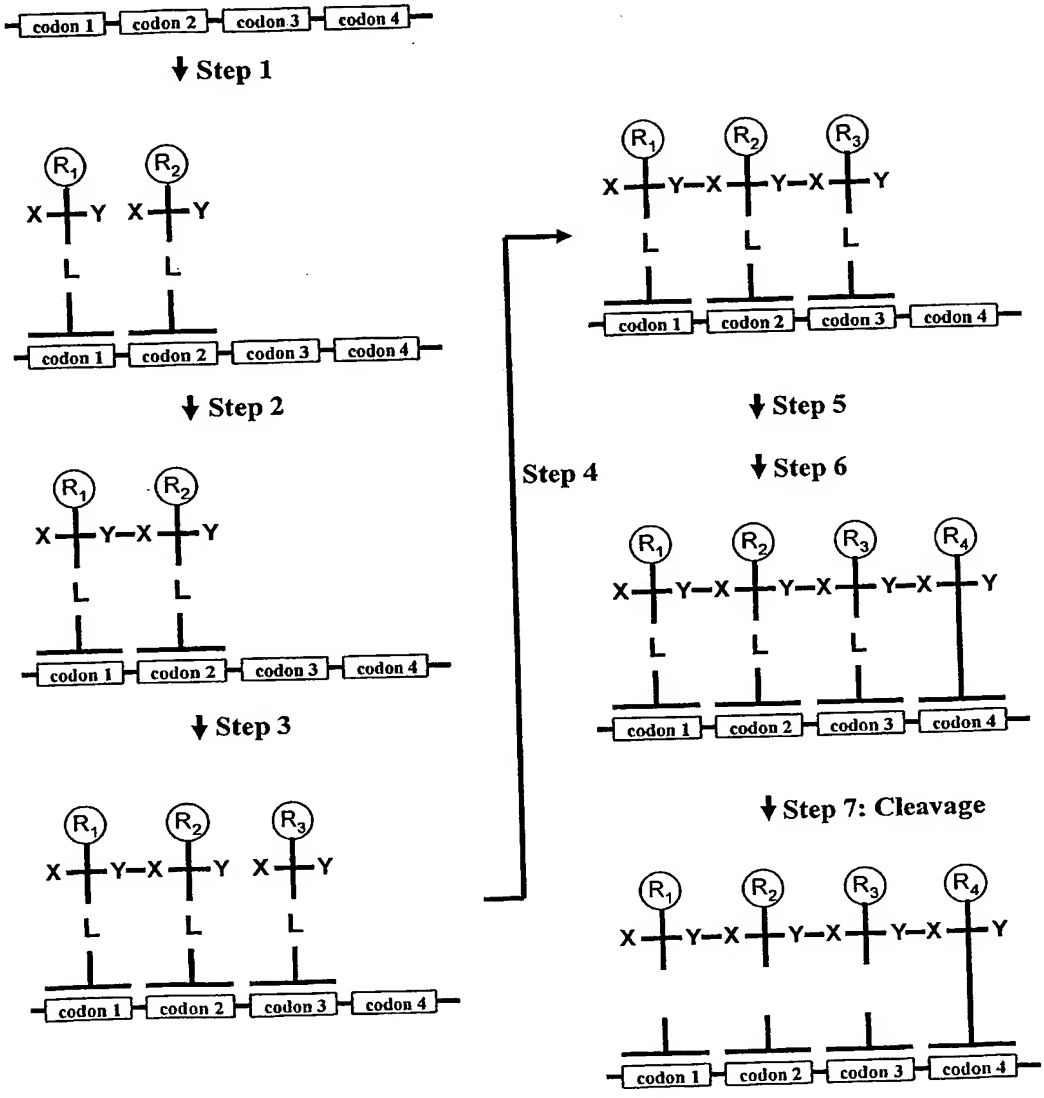


Fig. 8, example 1. Light-induced reaction between symmetric building blocks: Coumarin derivatives.

A

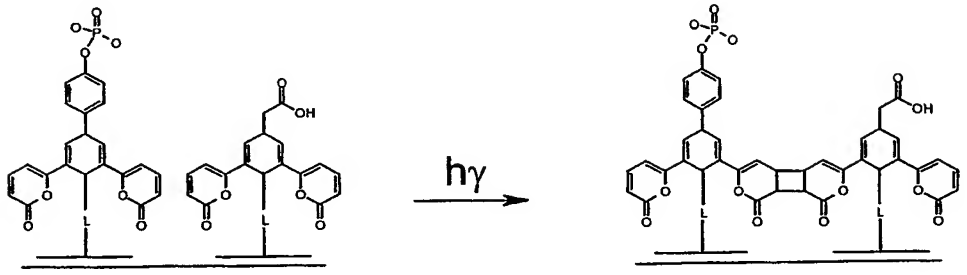


**Fig. 9. Templated synthesis of a polymer by simultaneous reaction and cleavage.**

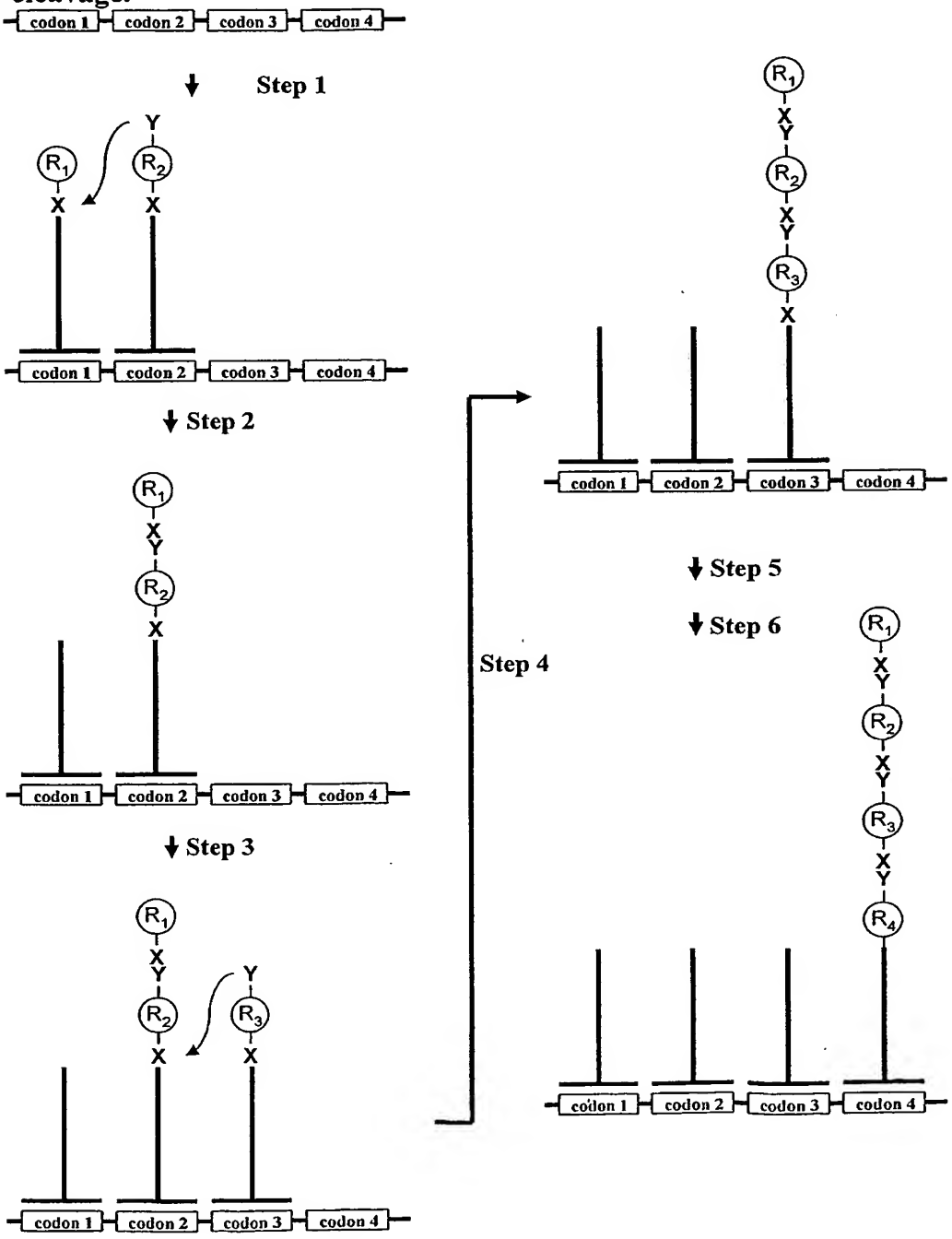


**Fig. 10. Templated synthesis of a mixed polymer by simultaneous reaction and cleavage.**

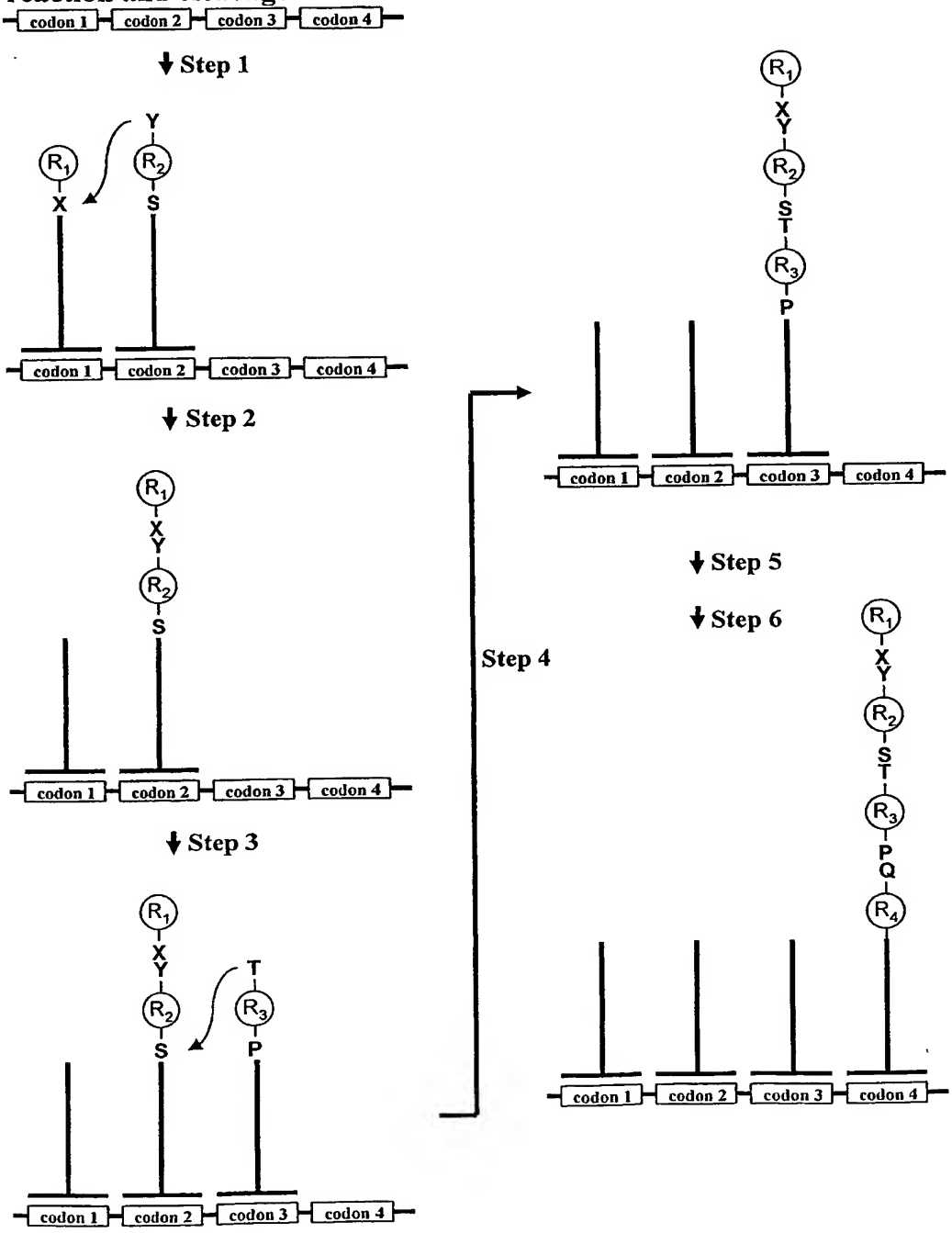


**Fig. 10, example 1. Simultaneous reaction and cleavage: Formation of (A) an alpha-peptide, and (C) a polyamine.**

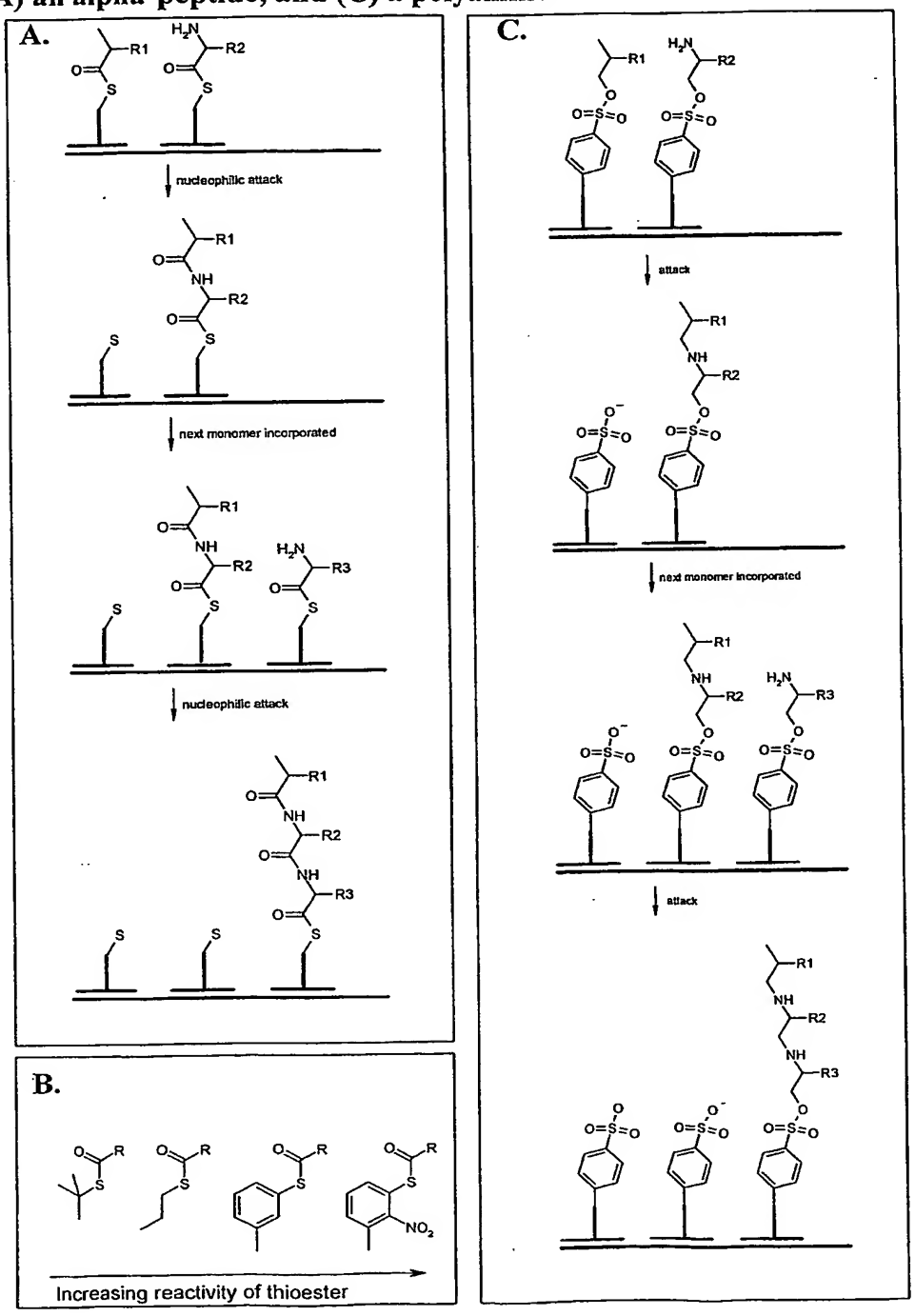


**Fig. 10, example 2. Simultaneous reaction and cleavage: Formation of (A) a peptoid or an alpha- or beta-peptide, and (B) a hydrazino peptide.**

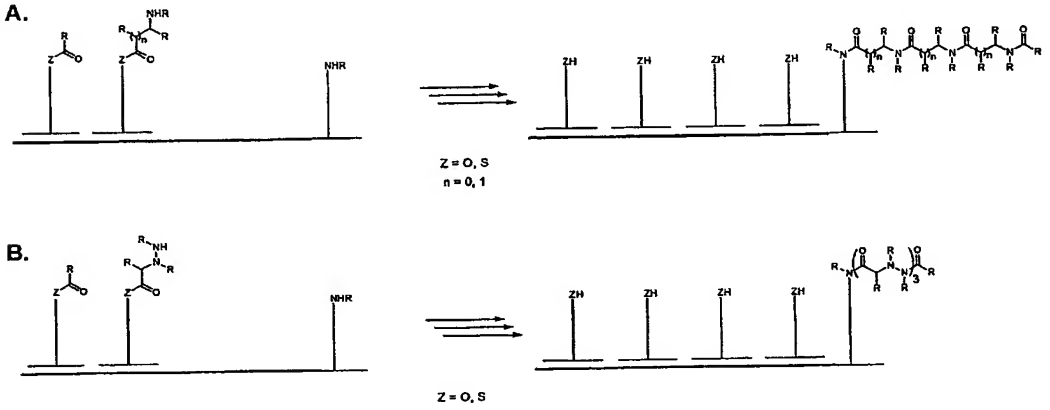


**Fig. 11. Templated synthesis of a polymer, using non-simultaneous reaction and cleavage.**

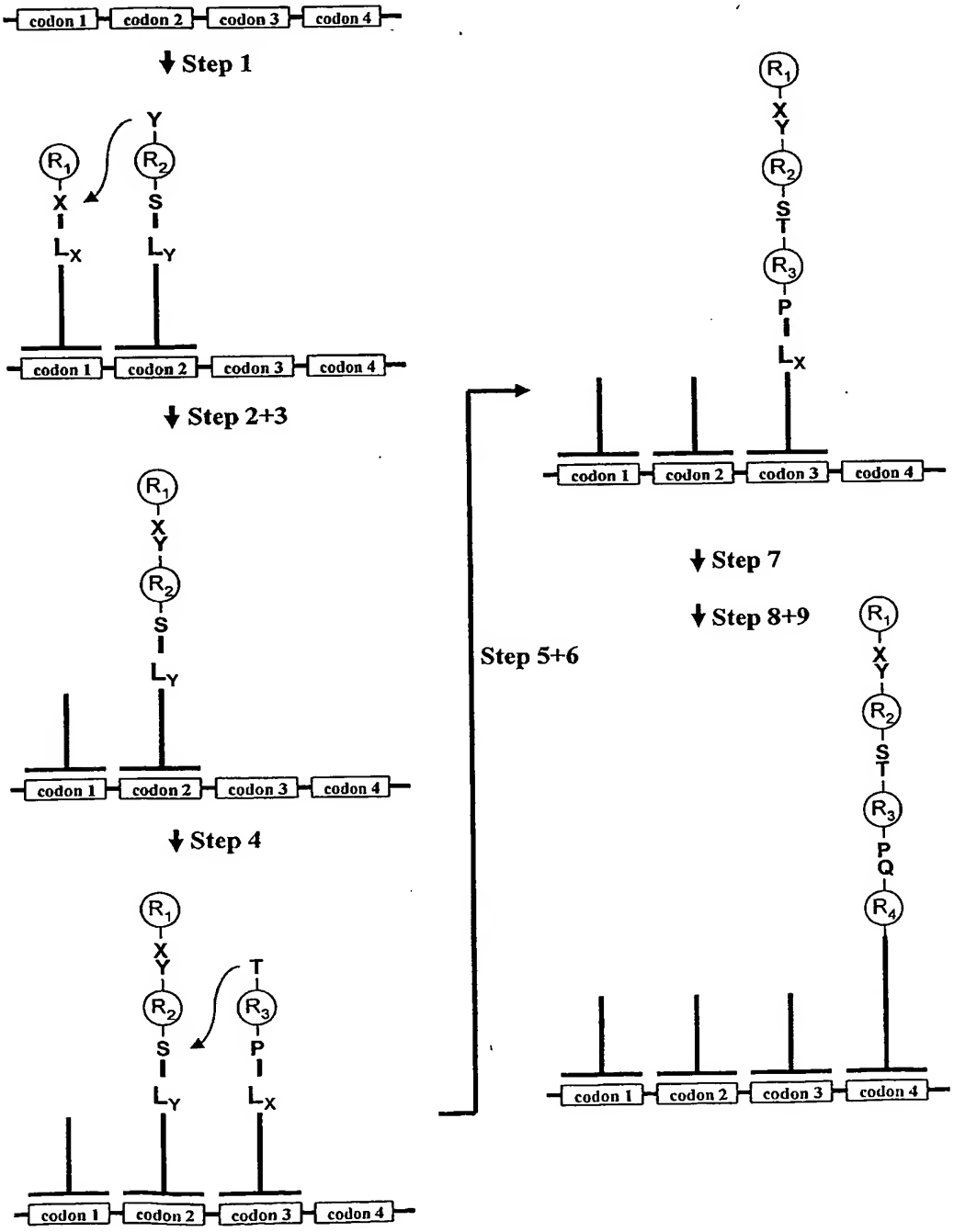


**Fig. 12. Activation of reactive group and release from anti-codon by ring opening.**

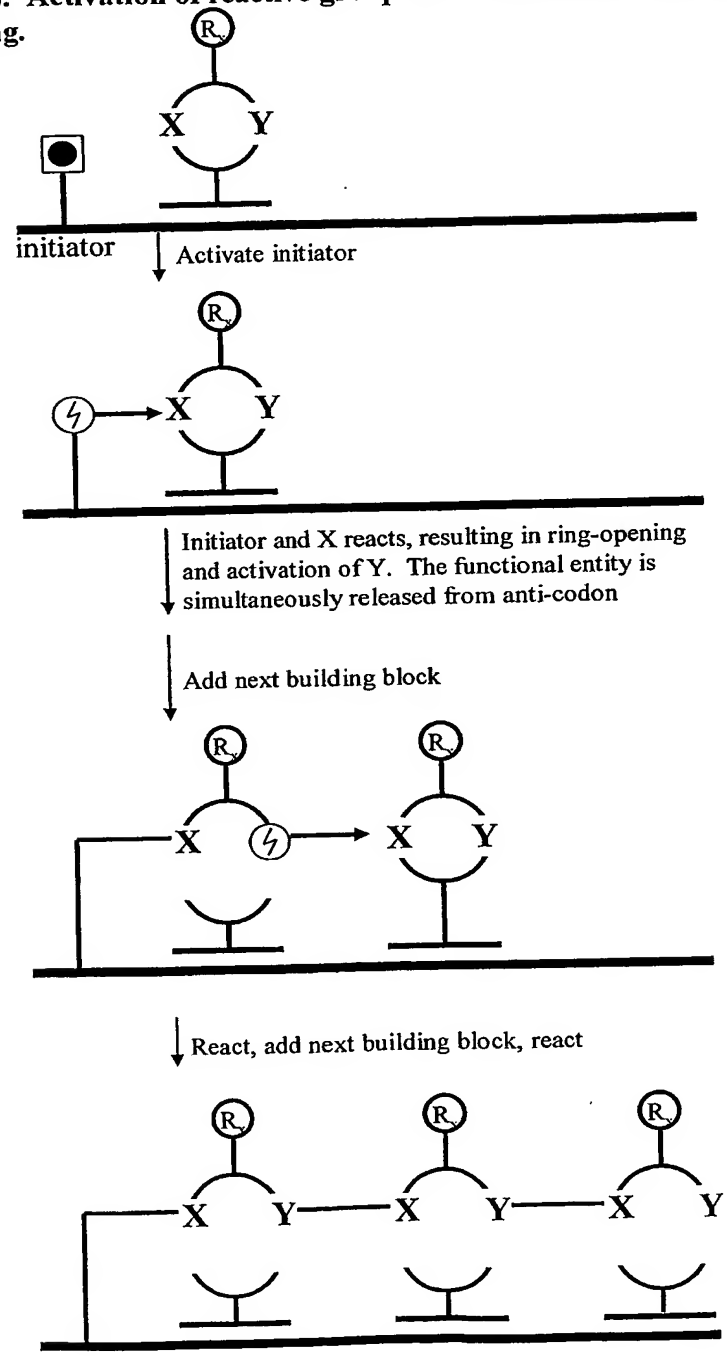


**Fig. 13. Symmetric fill-in reaction (symmetric XX building blocks).**

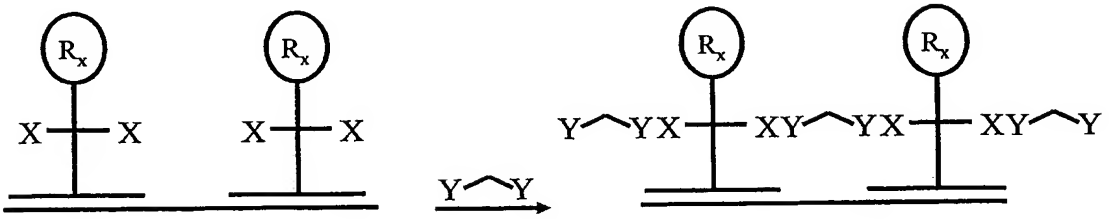



**Fig. 13, ex 1. Imine formation by fill-in.**

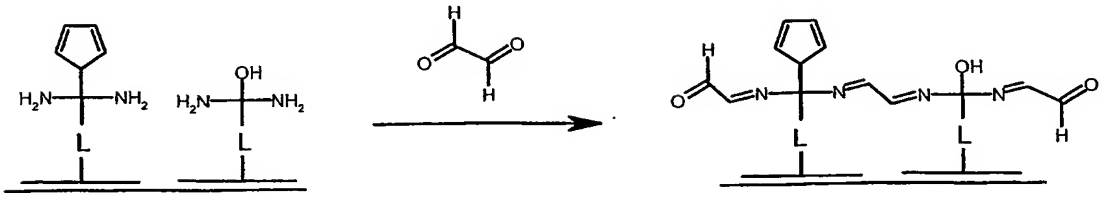


**Fig. 13, example 2. Amide formation.**

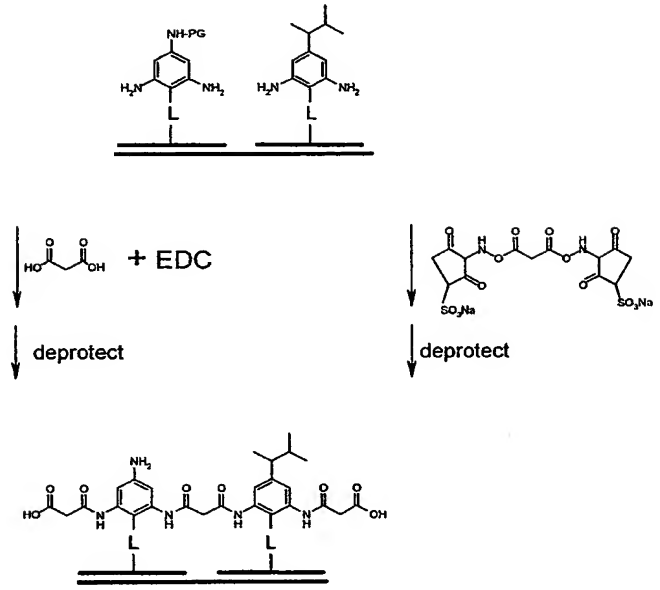



**Fig. 13, example 3. Urea formation**

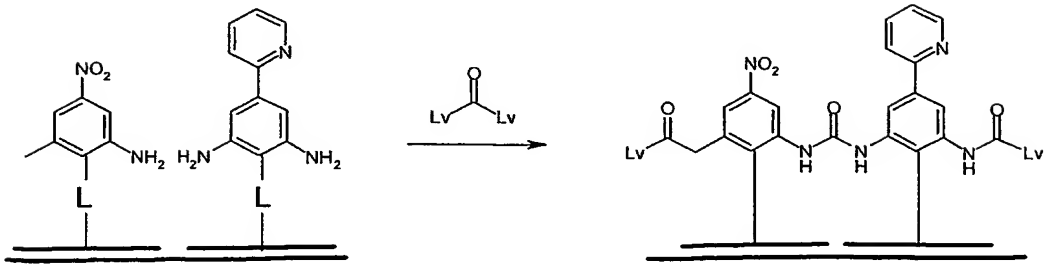
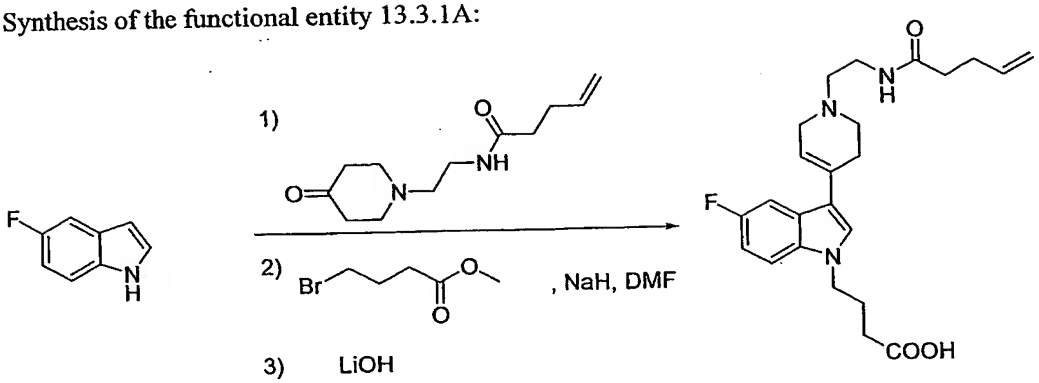


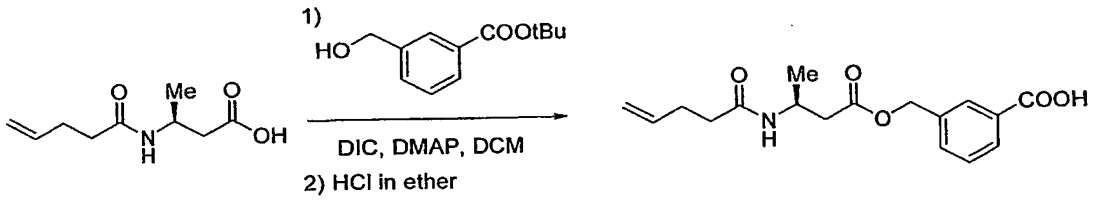
**Fig 13, ex 3.1 Urea formation**

Synthesis of the functional entity 13.3.1A:



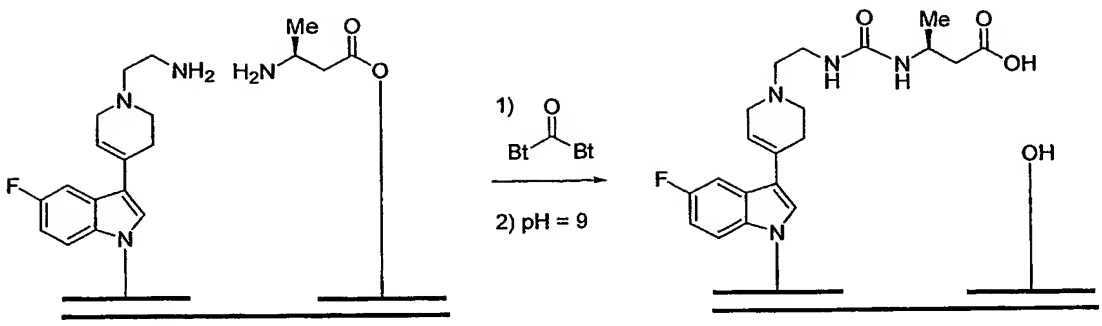

5-Fluoroindole (1eq) is dissolved in ethanol and treated with pent-4-enoic acid [2-(4-oxo-piperidin-1-yl)-ethyl]-amide (1.2 eq) and 2N KOH. The mixture is stirred o/n at reflux. The crude is evaporated and purified by silica gel filtration. The purified materiel is treated with methyl 3-bromobutanoate (1.2 eq) and NaH (1.5 eq) in DMF at rt. After 5 hours LiOH (10 eq) and water is added and the reaction mixture is stirred at rt o/n. The final product is purified by LC-MS and loaded on a DNA oligo containing an amino function.

Synthesis of the functional entity 13.3.1B:




3-Pent-4-enoylamino-butyric acid (1 eq) is treated with 3-hydroxymethyl-benzoic acid tert-butyl ester (1.2 eq), DIC (1.2 eq) and DMAP (0.2 eq) in DCM. The reaction mixture is stirred o/n at rt. The crude is evaporated and purified by silica gel filtration. The purified material is dissolved in diethyl ether and treated with HCl in diethyl ether. After stirring for 3 hours the mixture is evaporated and the crude material loaded on a DNA oligo containing an amino function.

Fill in experiment using functional entity 13.3.1A and 13.3.1B:




The two loaded oligos are mixed with a template oligo in in hepes buffer (pH = 7.5) and 100 mM NaCl. 1,1'-Carbonylbisbenzotriazole (0.1M in MeOH) is added and the mixture is left at rt for 4 hours. pH is then adjusted to 9 and the mixture is left at rt o/n.

**Fig. 13, ex 4. Chiral and non-chiral templated molecule**

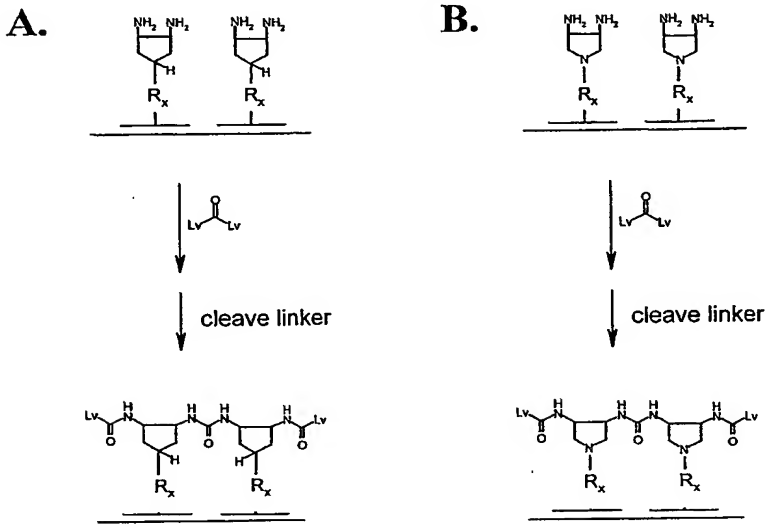


**Fig. 13, ex 5. Symmetric fill-in: Formation of a phophodiester bond.**

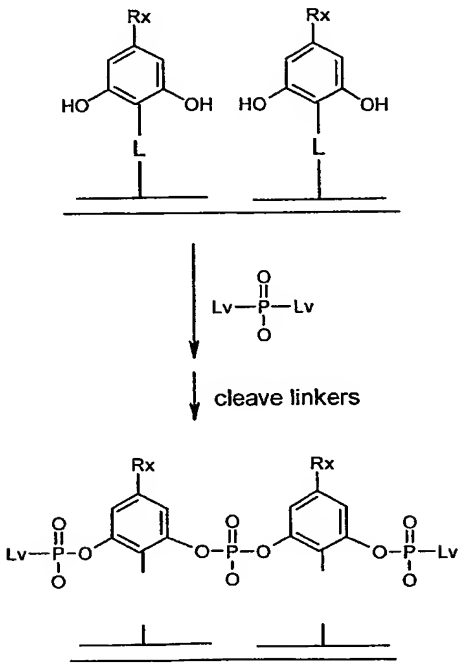


**Fig. 13, ex 6. Fill-in: Phophodiester formation with one reactive group in each building block**

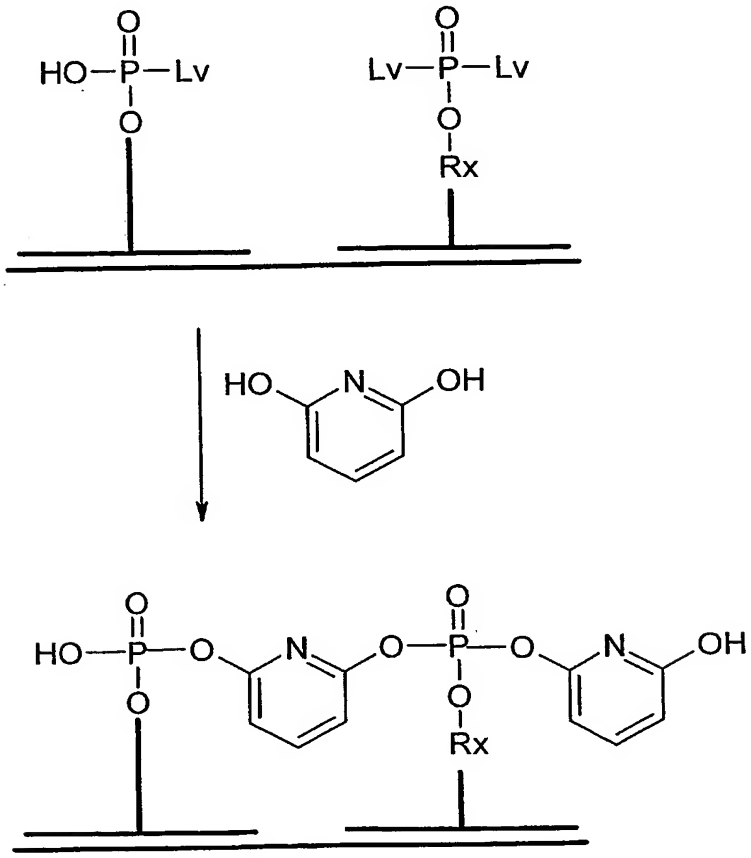


**Fig. 13, ex 7. Pericyclic reaction.**

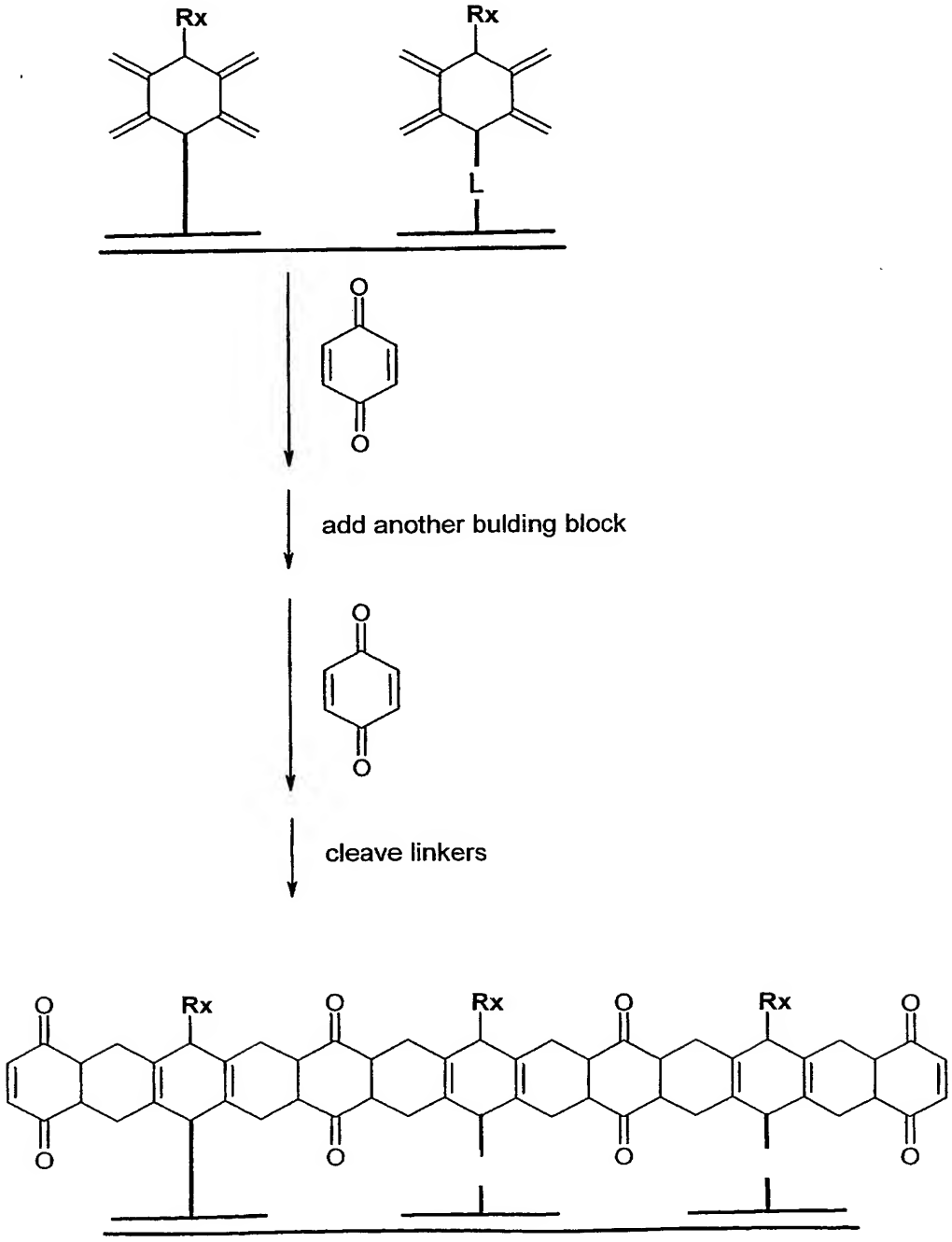
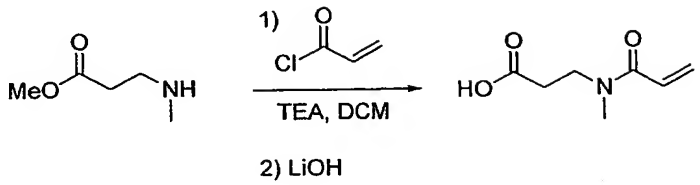


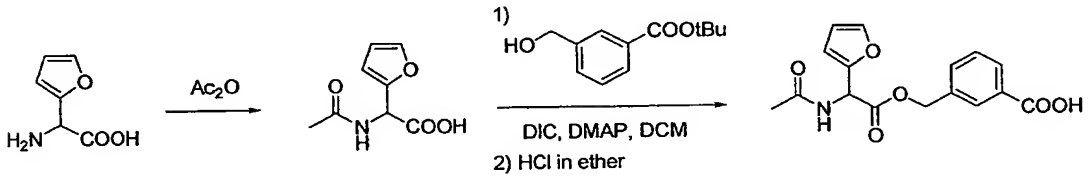
Figure 13, ex 7.1 Pericyclic reaction

Synthesis of the functional entity 13.7.1A:



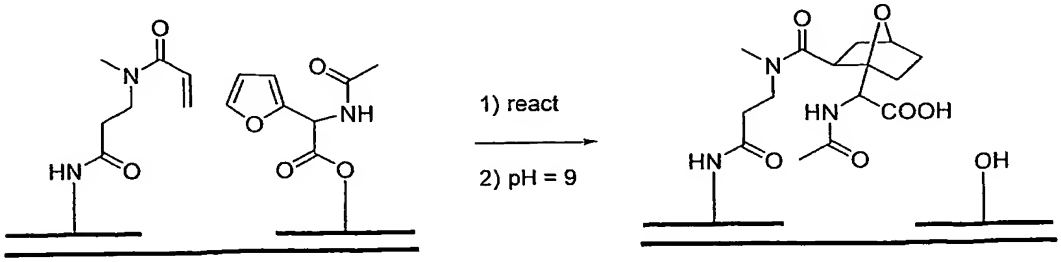

3-Methylamino-propionic acid methyl ester (1eq) is dissolved in DCM and triethylamine (2 eq). The mixture is cooled to 0° and treated with acryloyl chloride (1.5 eq). After 2 hours the reaction mixture is evaporated, redissolved in THF and treated with LiOH (10 eq) and water. The mixture is left at rt for 3 hours. The crude is extracted with EtOAc (2x). The combined organic phases are dried over MgSO4 and evaporated. The product is purified by LC-MS and loaded on a DNA oligo containing an amino function.

Synthesis of the functional entity 13.7.1B:




Amino-furan-2-yl-acetic acid (1eq) is treated with acetic anhydride (3 eq) at rt for 1 hour. The crude is evaporated and the product purified by LC-MS and then treated with 3-hydroxymethyl-benzoic acid tert-butyl ester (1.2 eq), DIC (1.2 eq) and DMAP (0.2 eq) in DCM. The reaction mixture is stirred o/n at rt. The crude is evaporated and purified by silica gel filtration. The purified material is dissolved in diethyl ether and treated with HCl in diethyl ether. After stirring for 3 hours the mixture is evaporated and the crude material loaded on a DNA oligo containing an amino function.

Pericyclic reaction experiment using functional entity 13.7.1A and 13.7.1B:



The two loaded oligos are mixed with a template oligo in in hepes buffer (pH = 7.5) and 100 mM. The mixture is left at rt for 4 hours. pH is then adjusted to 9 and the mixture is left at rt o/n.

**Fig. 13. "Fill-in" reaction (asymmetric XS monomers).**

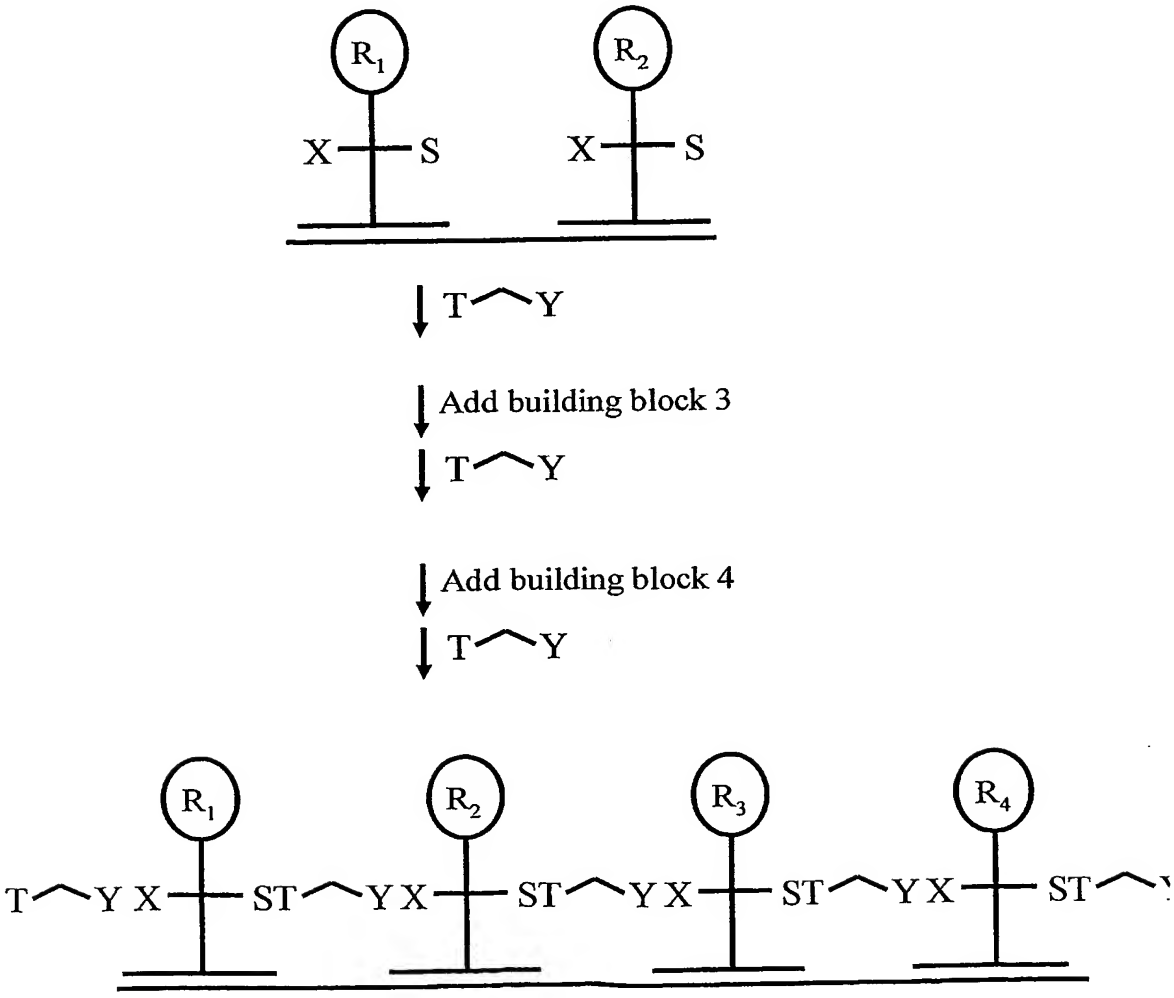


**Fig. 14, example 1.**

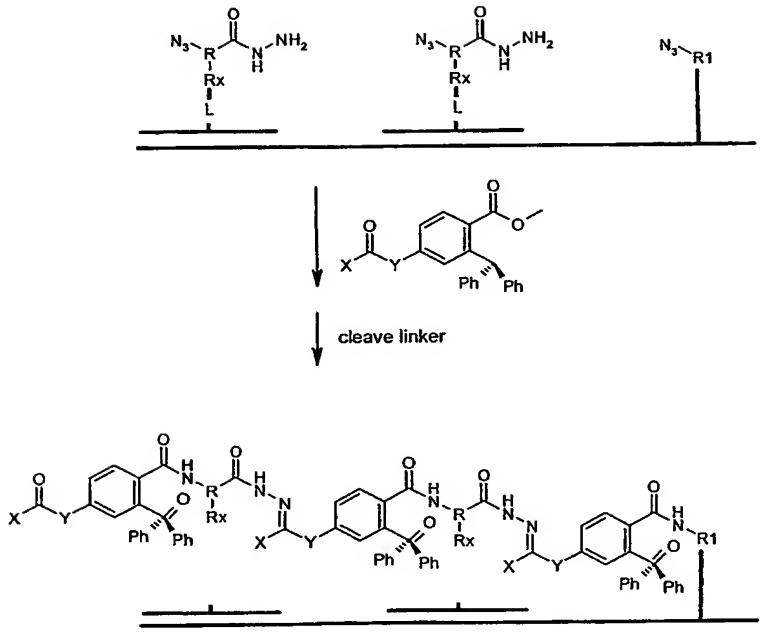


**Fig. 15. Templated synthesis of a non-linear molecule.**

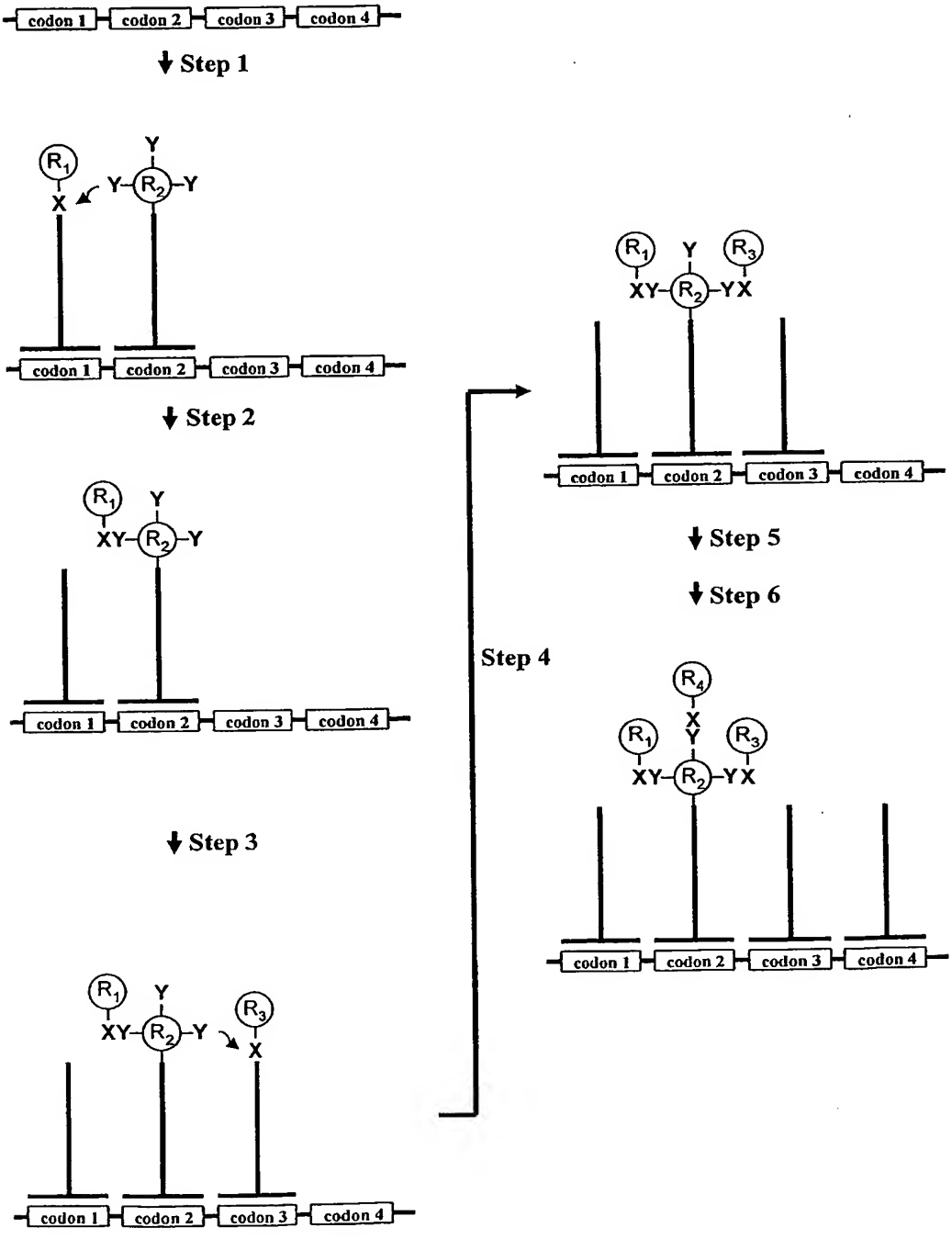


**Fig. 16. Templated synthesis of a non-linear molecule, employing reactive groups of different classes, and non-simultaneous reaction and cleavage.**

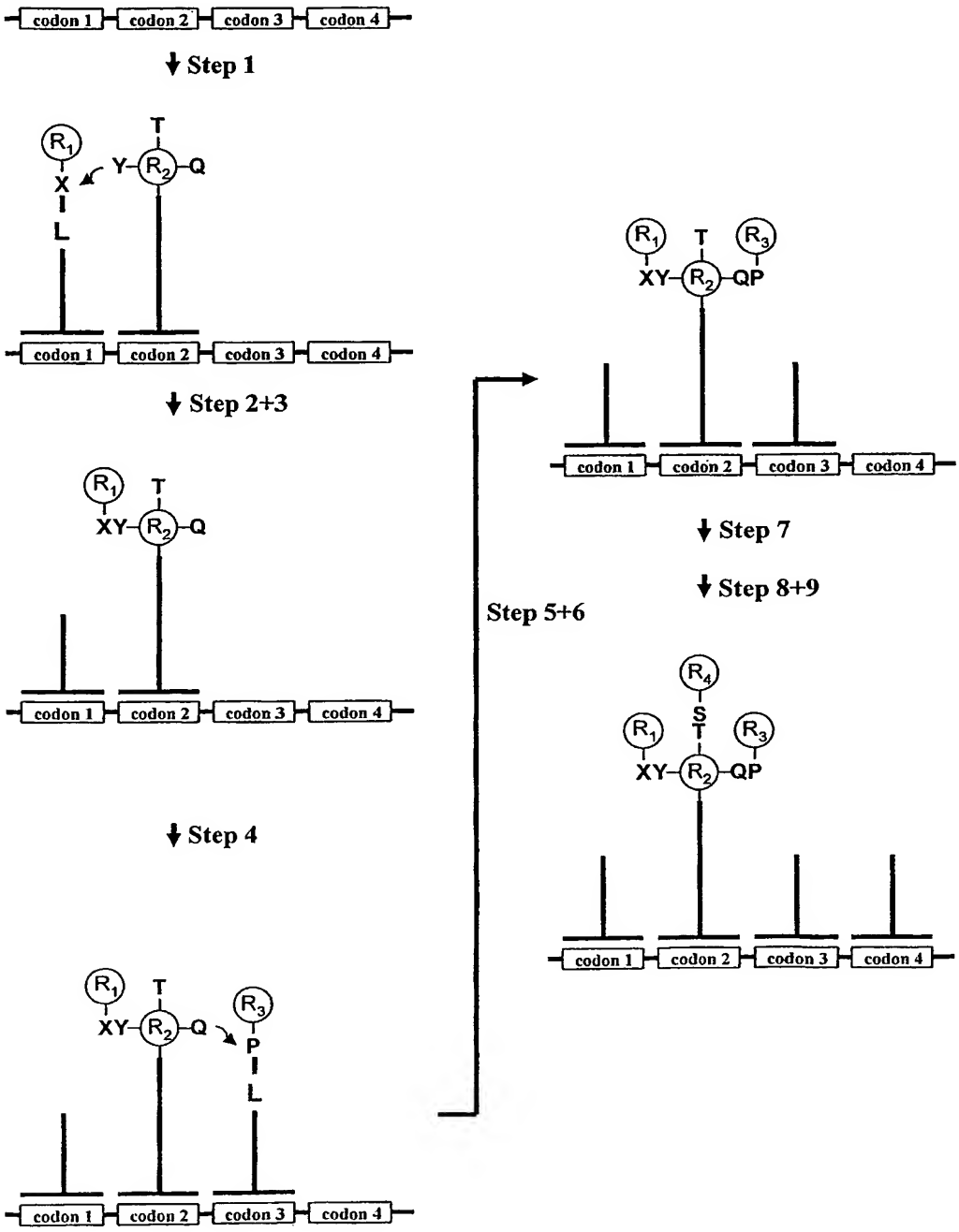


**Fig. 17. Migrating scaffold. Templated synthesis of a non-linear molecule, by exploiting the increased proximity effect that arises from a "migrating" scaffold.**

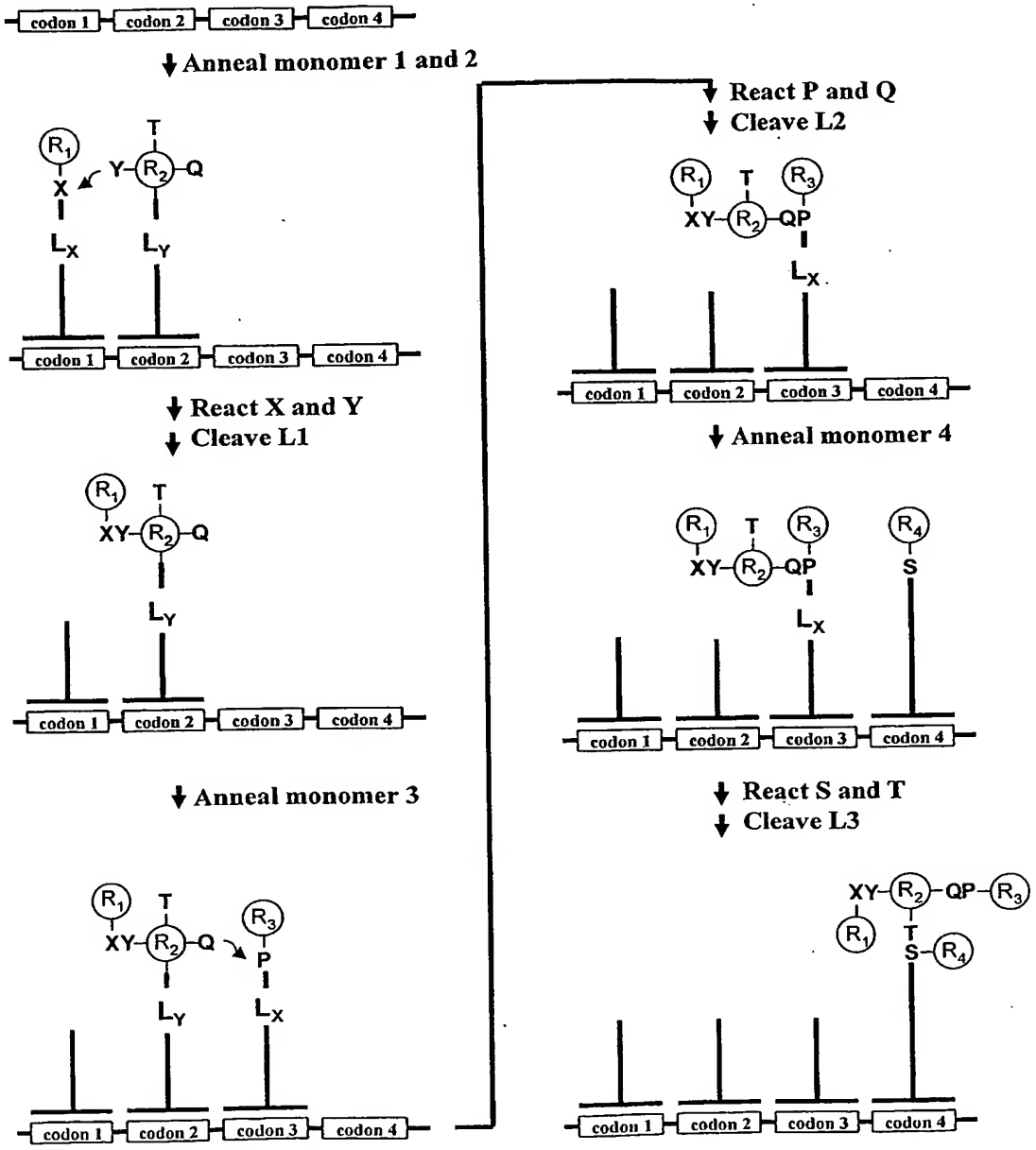
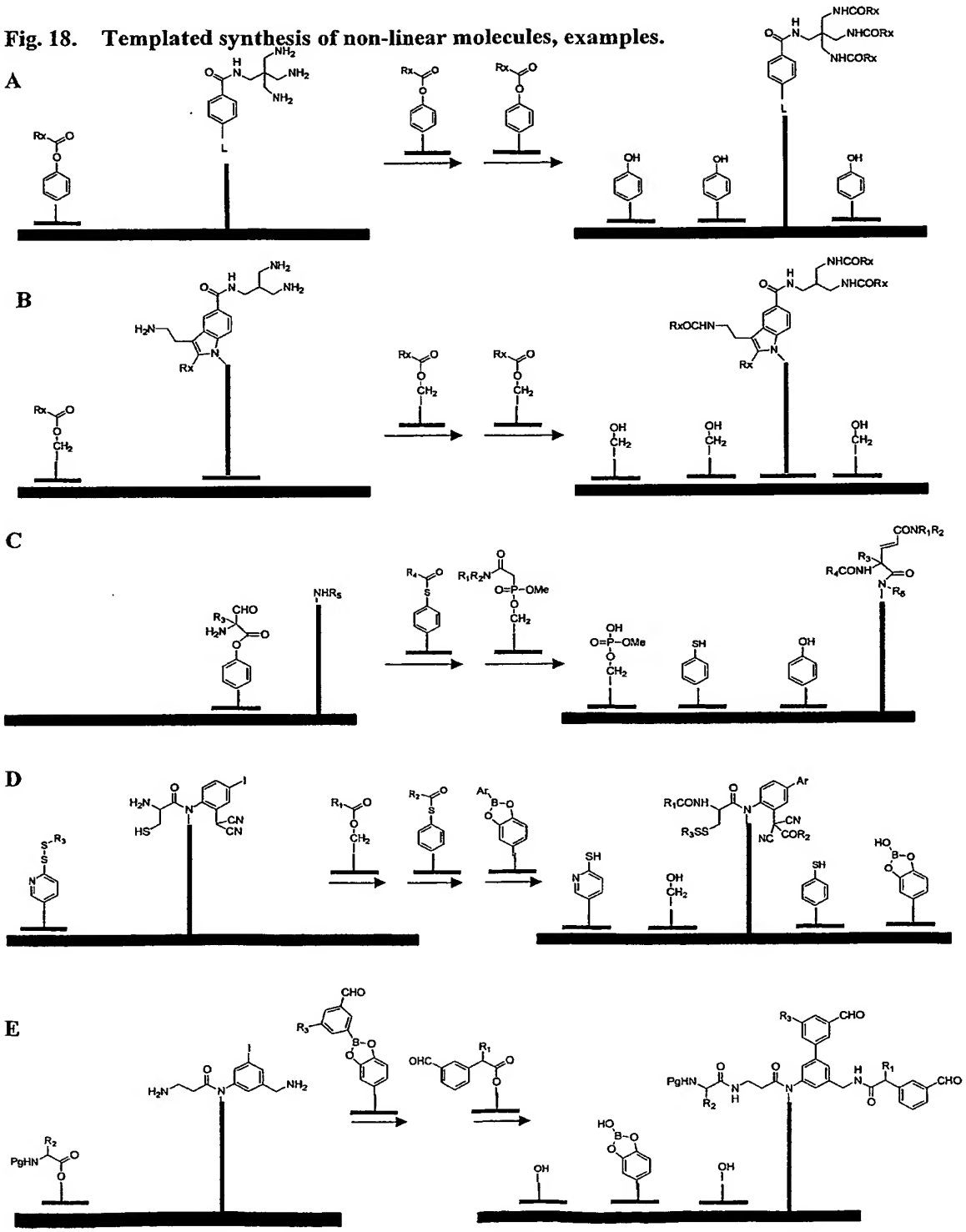


**Fig. 18. Templated synthesis of non-linear molecules, examples.**



F

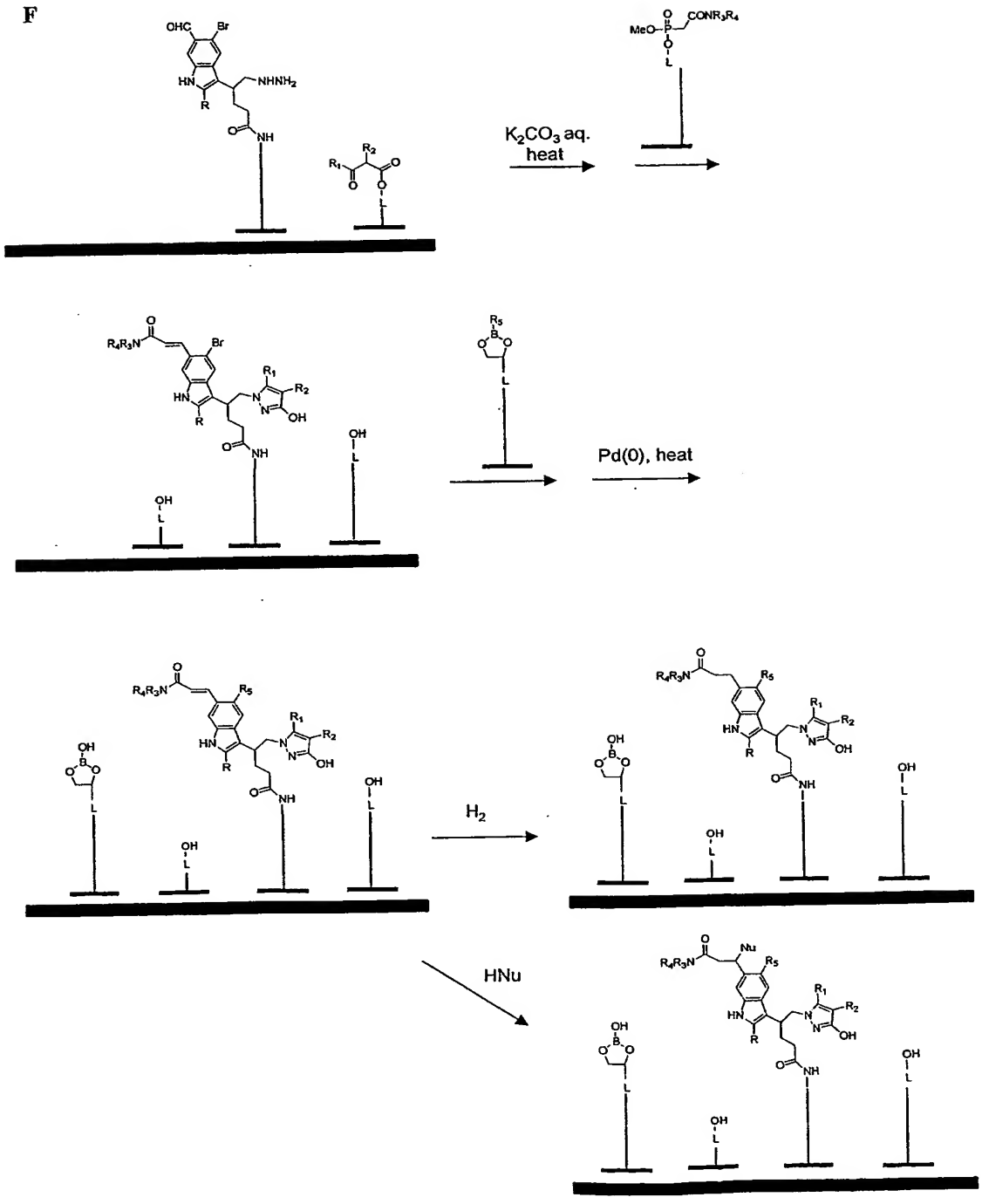


**Fig. 19. Templated synthesis in whiche the reaction step is performed under conditions where specific annealing of building block to template is inefficient.**

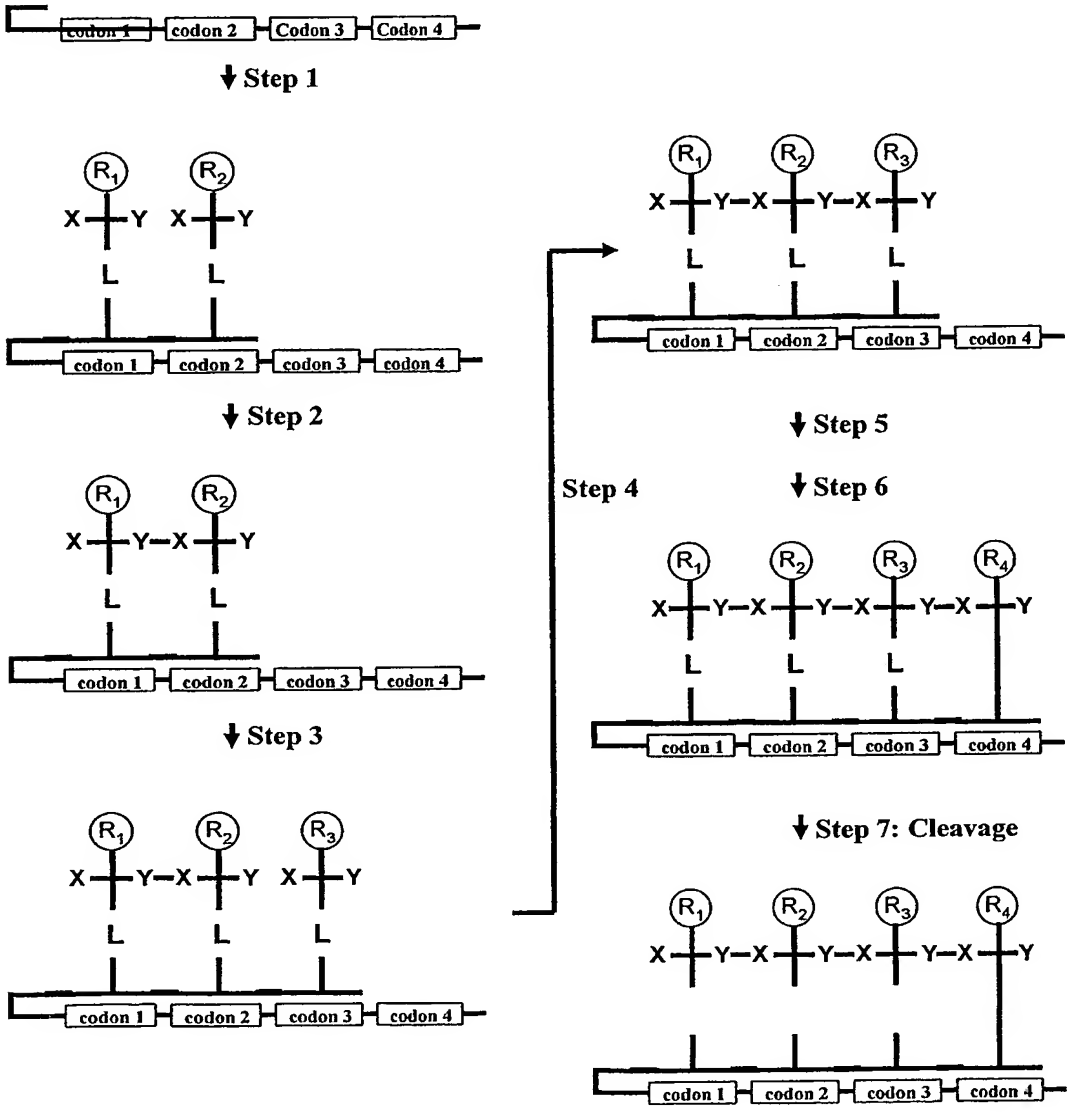
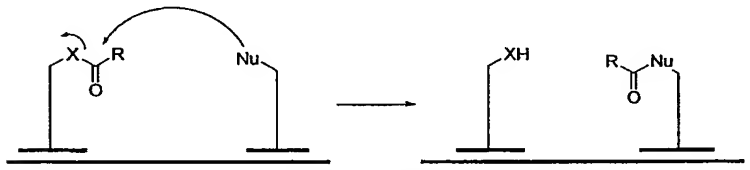


**Fig. 20. Reaction types allowing simultaneous reaction and activation.**

**Nucleophilic substitution using activation of electrophiles**

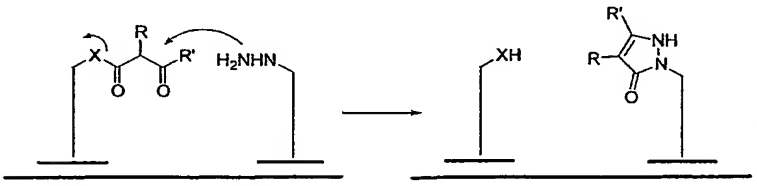
**A. Acylating monomer building blocks - principle**



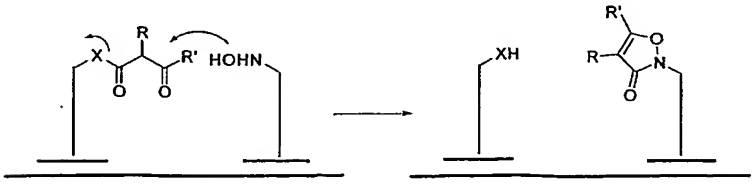

X = O, S Nu = Oxygen- , Nitrogen- , Sulfur- and Carbon Nucleophiles

**B. Acylation**
**Amide formation by reaction of amines with activated esters**


**C. Acylation**
**Pyrazolone formation by reaction of hydrazines with β–Ketoesters**

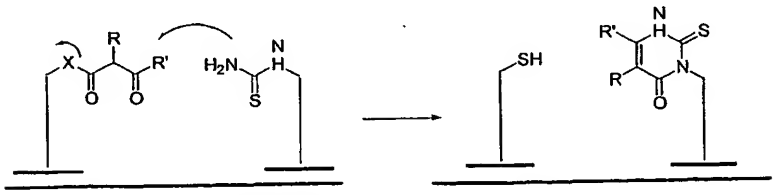



**D. Acylation**
**Isoxazolone formation by reaction of hydroxylamines with β–Ketoesters**



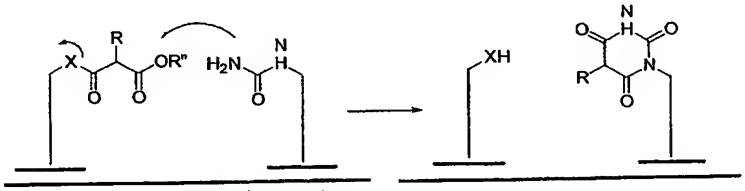
**E. Acylation**

**Pyrimidine formation by reaction of thioureas with β–Ketoesters**



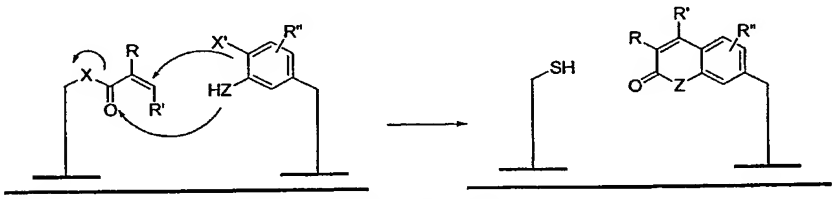
**F. Acylation**

**Pyrimidine formation by reaction of ureas with Malonates**



**G. Acylation**

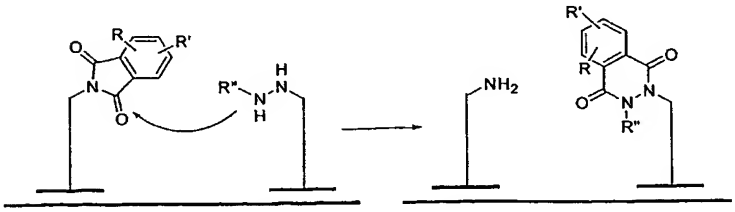
**Coumarine or quinolinon formation by a Heck reaction followed by a nucleophilic substitution**



X = O,S X' = Halogen, OTf, OMs Z = O, NH

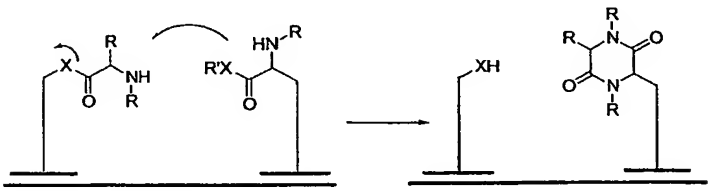
**H. Acylation**

**Phthalhydrazide formation by reaction of Hydrazines and Phthalimides**



**I. Acylation**

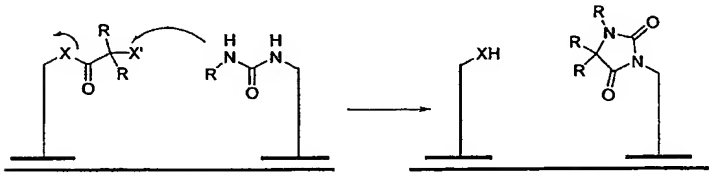
**Diketopiperazine formation by reaction of Amino Acid Esters**



X = O, S R' = H, R

**J. Acylation**

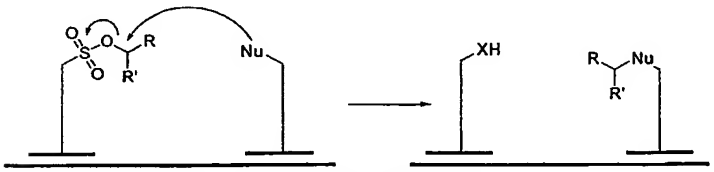
**Hydantoin formation by reaction of Urea and α-substituted Esters**



X = O, S X' = Hal, OTos, OMs, etc.

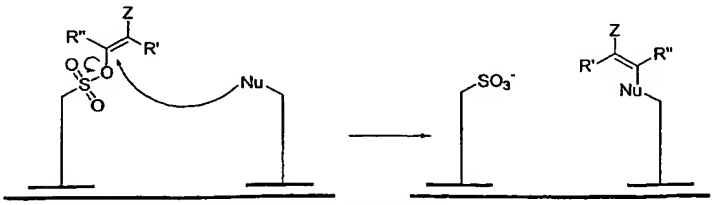
**K. Alkylating monomer building blocks - principle**

**Alkylated compounds by reaction of Sulfonates with Nucleofiles**



Nu = Oxygen- , Nitrogen- , Sulfur- and Carbon Nucleophiles

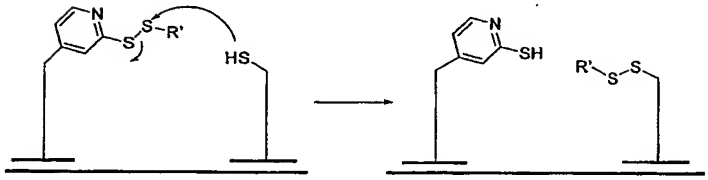
**L. Vinylating monomer building blocks - principle**



Z = CN, COOR, COR, $NO_2$, $SO_2R$, S(=O)R, $SO_2NR_2$, F
Nu = Oxygen- , Nitrogen- , Sulfur- and Carbon Nucleophiles

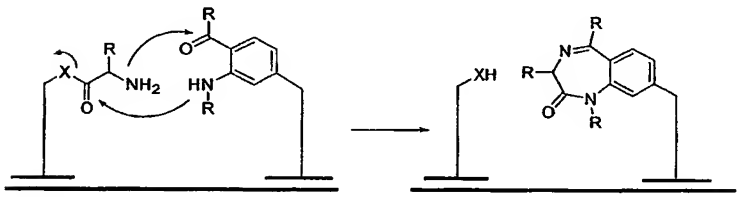
**M. Heteroatom electrophiles**

**Disulfide formation by reaction of Pyridyl disulfide with Mercaptanes**



**N. Acylation**

**Benzodiazepinone formationby reaction of Amino Acid Esters and Amino Ketones**

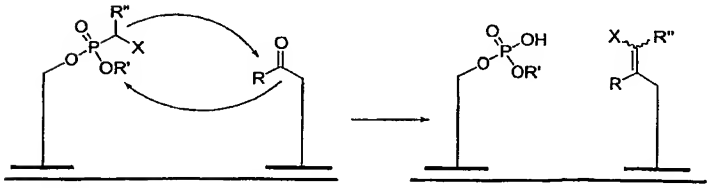


X = O, S

## Addition to carbon-hetero multiple bonds

**O. Wittig/Horner-Wittig-Emmons reagents**

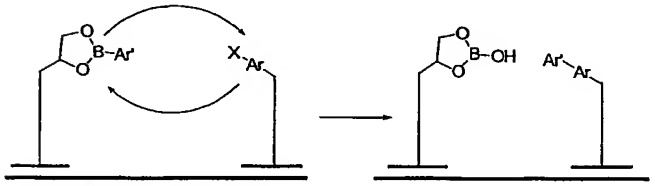
**Substituted alkene formation by reaction of Phosphonates with Aldehydes or Ketones**



X = EWG

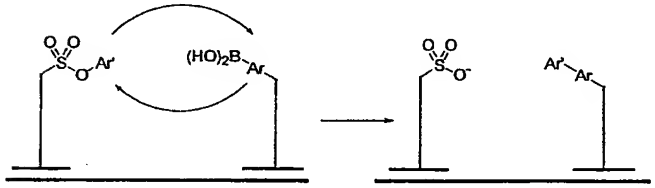
## Transition metal catalysed reactions

**P. Arylation**
**Biaryl formation by the reaction of Boronates with Aryls or Heteroaryls**

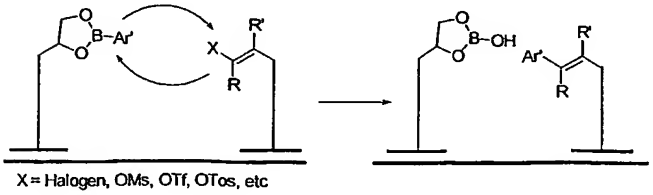



X = Halogen, OMs, OTf, OTos, etc

**Q. Arylation**
**Biaryl formation by the reaction of Boronates with Aryls or Heteroaryls**

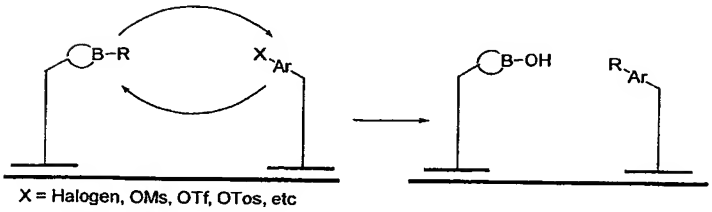



**R. Arylation**
**Vinylarene formation by the reaction of alkenes with Aryls or Heteroaryls**

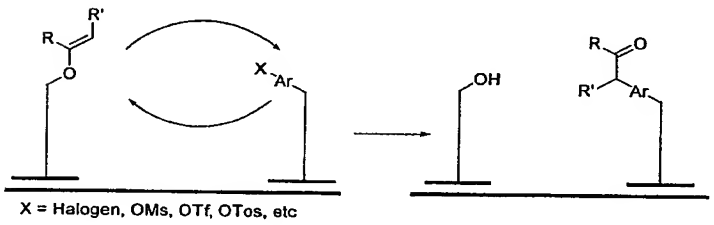



X = Halogen, OMs, OTf, OTos, etc

**S. Alkylation**
**Alkylation of arenes/hetarens by the reaction with Alkyl boronates**

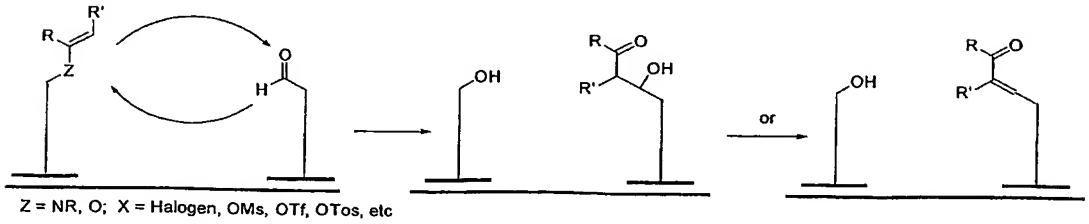



**T. Alkylation**
**Alkylation of arenas/hetarenes by reaction with enolethers**



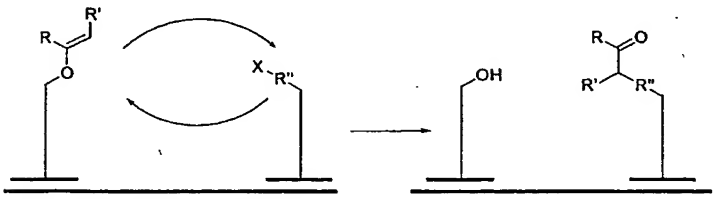

## Nucleophilic substitution using activation of nucleophiles

**U. Condensations**
**Alkylation of aldehydes with enolethers or enamines**



**V. Alkylation**

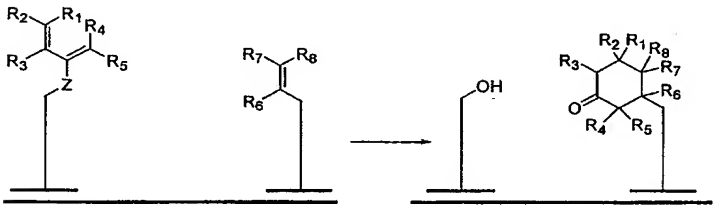
**Alkylation of aliphatic halides or tosylates with enolethers or enamines**




X = Halogen, OMs, OTf, OTos, etc

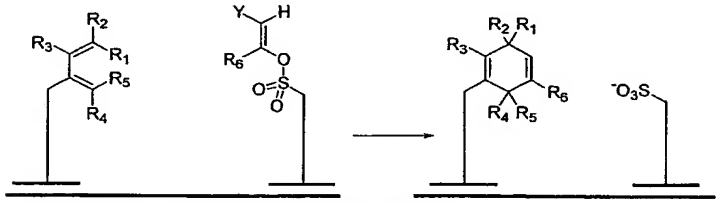
## Cycloadditions

**W. [2+4] Cycloadditions**



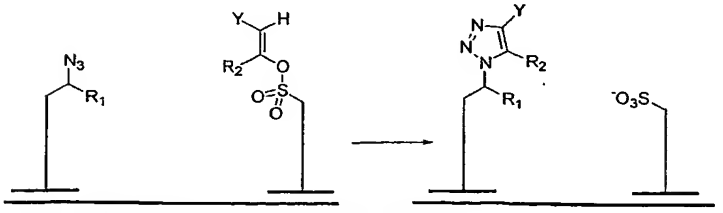

Z = O, NR

**X. [2+4] Cycloadditions**



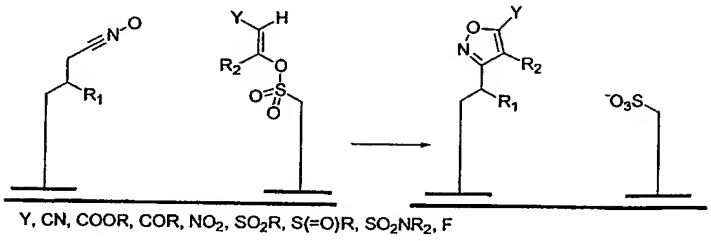

Y, CN, COOR, COR, $NO_2$, $SO_2R$, S(=O)R, $SO_2NR_2$, F

**Y. [3+2] Cycloadditions**




Y, CN, COOR, COR, $NO_2$, $SO_2R$, S(=O)R, $SO_2NR_2$, F

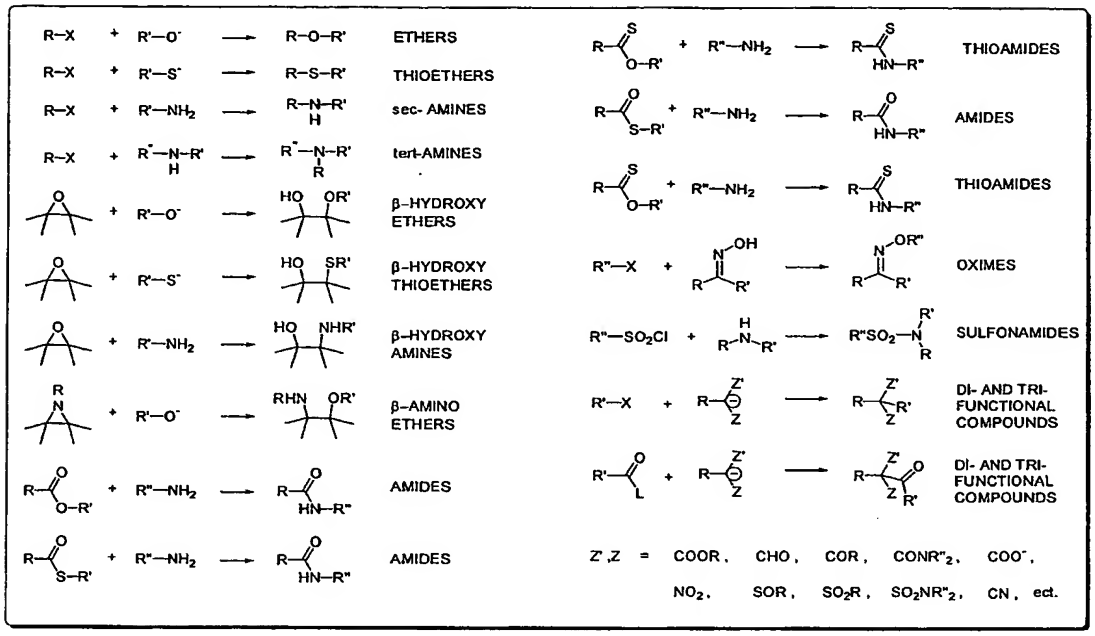
**Z. [3+2] Cycloadditions**



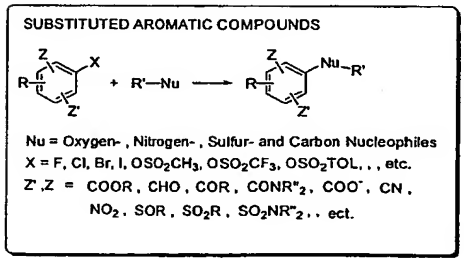

Y, CN, COOR, COR, $NO_2$, $SO_2R$, S(=O)R, $SO_2NR_2$, F

**Fig. 21. Pairs of reactive groups X,Y and the resulting bond XY.**

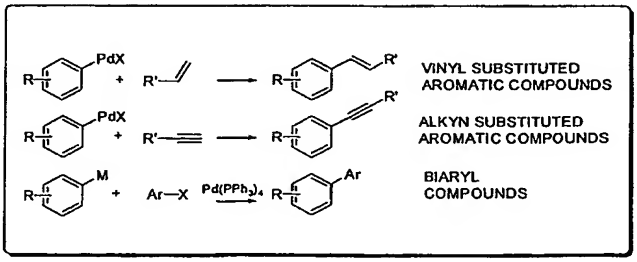
**Nucleophilic substitution reaction**



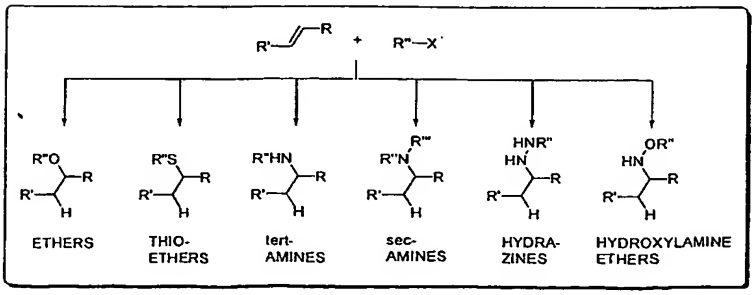

**Aromatic nucleophilic substitution**

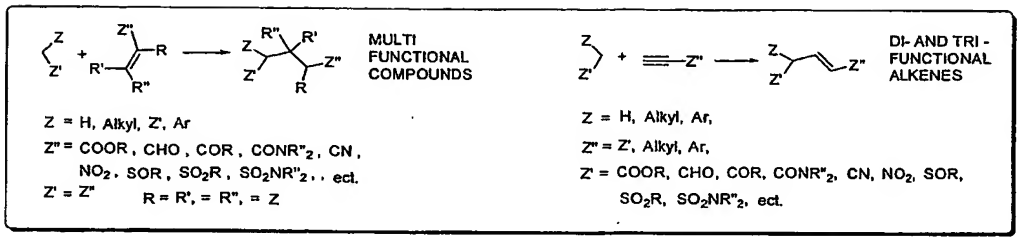



**Transition metal catalysed reactions**

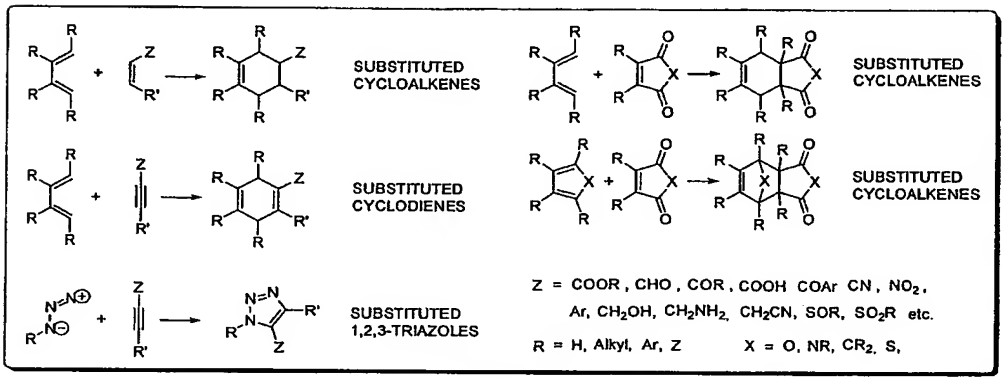



**Addition to carbon-carbon multiplebonds**






## Cycloaddition to multiple bounds




## Addition to carbon-hetero multiple bonds

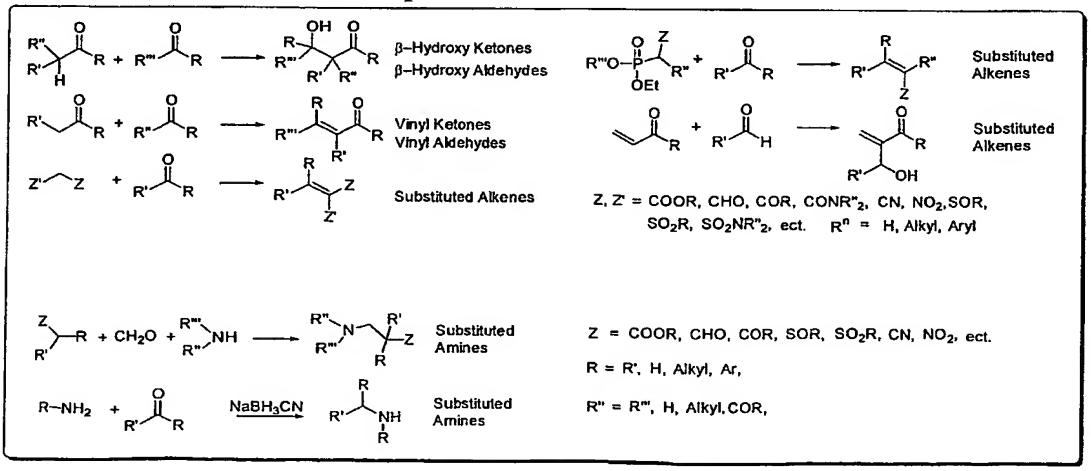
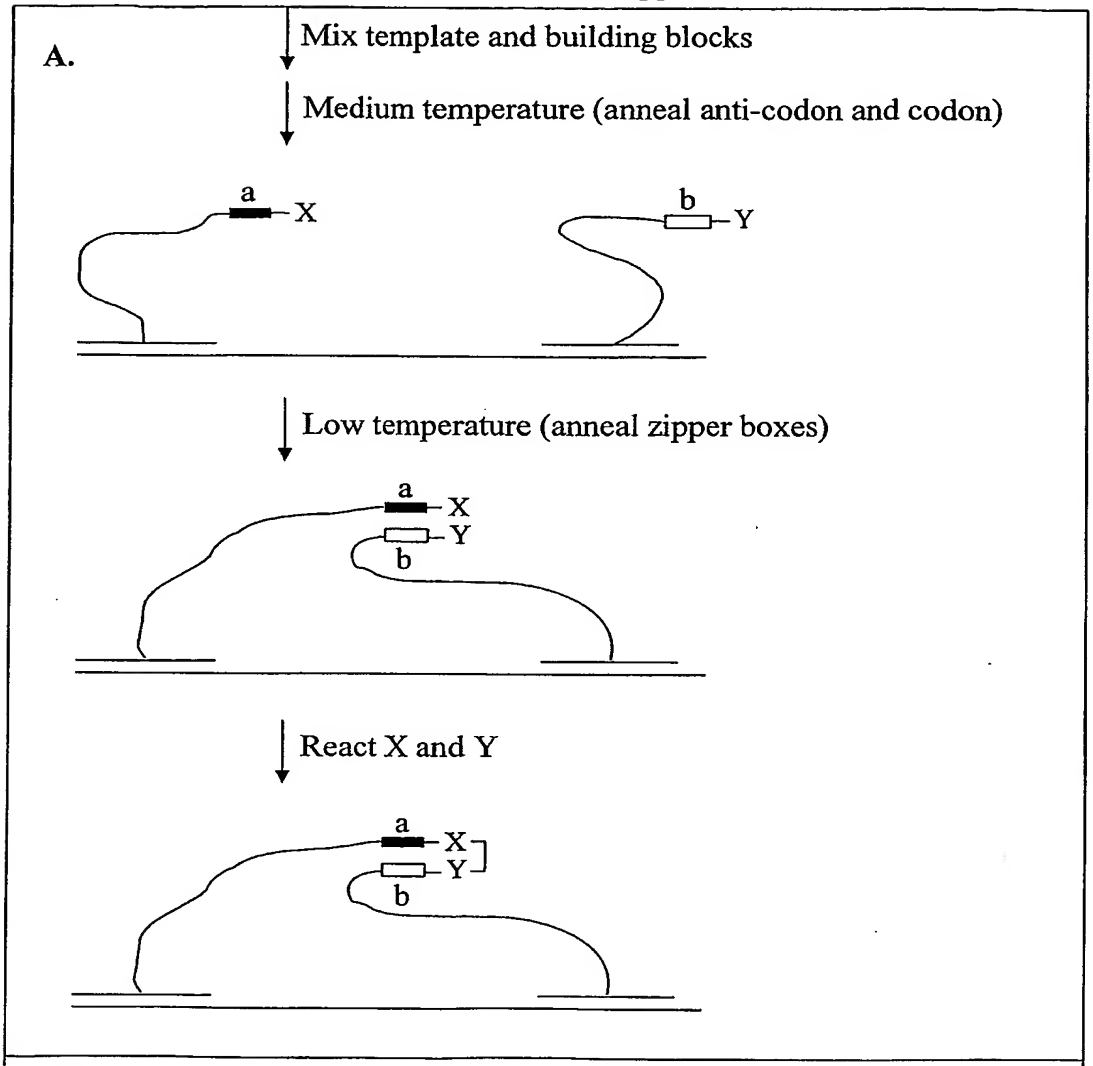


**Fig. 22. Increasing the proximity effect: Zipper box.**




**B.**

**Oligo1**

5'-CGACCTCTGGATTGCATCGGTCATGGCTGACTGTCCGTCGAATGTGTCCAGTTACX

Annealing region — Linker region — Zipper region

**Oligo2**

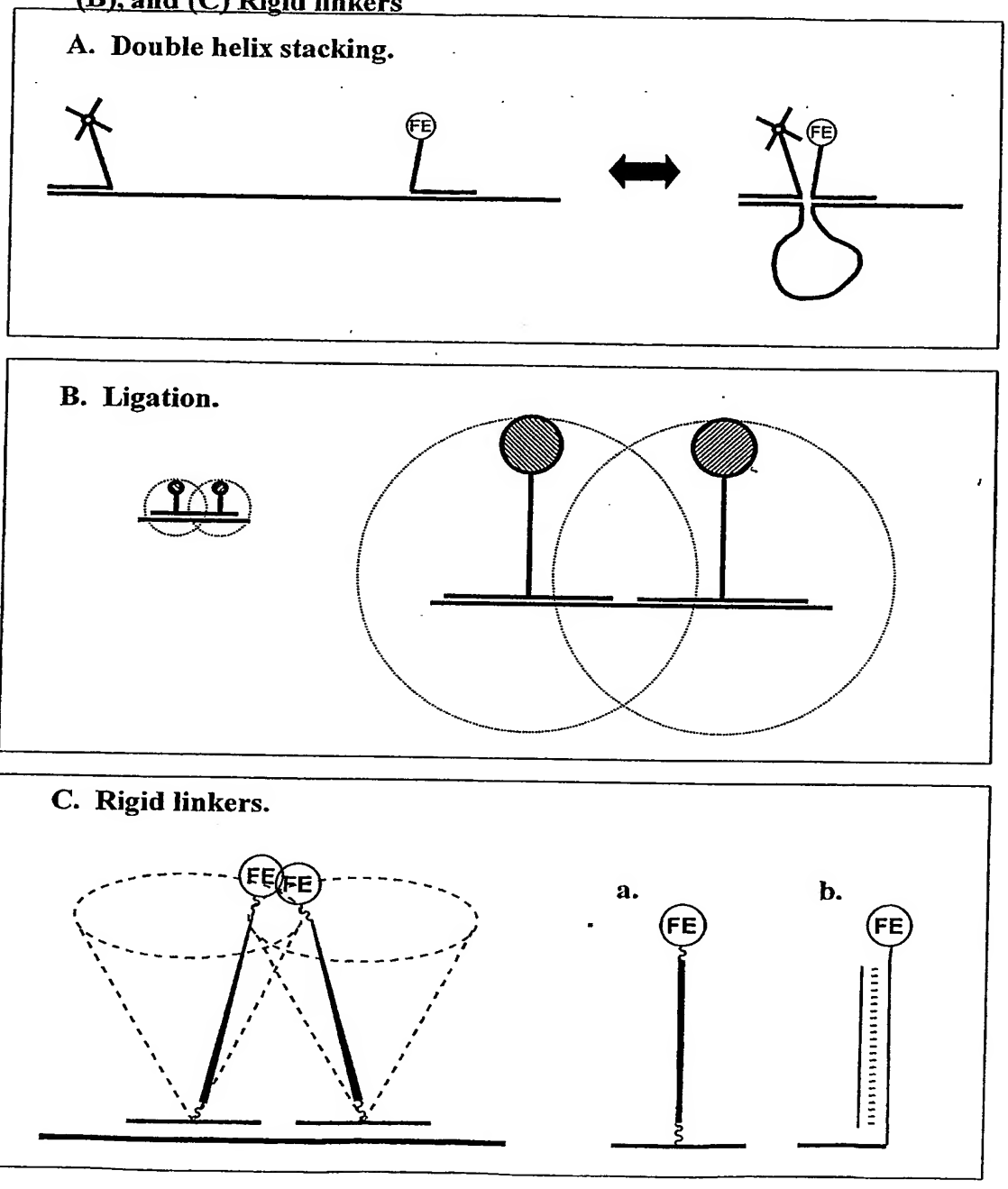
5'-ZGTAACACCTGTGTAAGCTGCCTGTCAGTCGGTACTGACCTGTCGAGCATCCAGC

Zipper region — Linker region — Annealing region

X= Carboxy-dT Glen Research

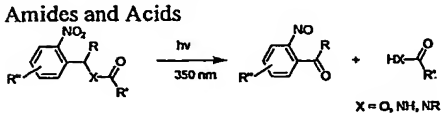
Z= Amino-Modifier Glen C6 dT Glen Research

**Fig. 23. Increasing the proximity effect: Helix Stacking (A), Ligation (B), and (C) Rigid linkers**

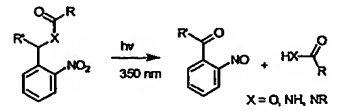


## Fig. 24. Cleavable Linkers

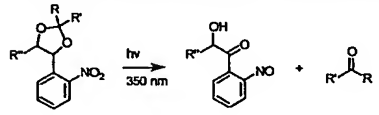
A. Linker for the formation of Ketones, Aldehydes, Amides and Acids



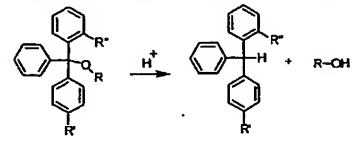

B. Linker for the formation of Ketones, Amides and Acids



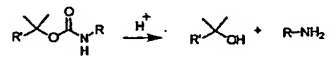

C. Linker for the formation of Aldehydes and Ketones



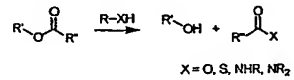

D. Linker for the formation of Alcohols and Acids



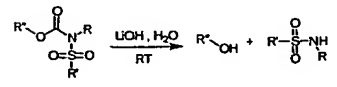

E. Linker for the formation of Amines and Alcohols



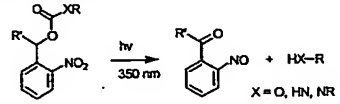

F. Linker for the formation of Esters, Thioesters , Amides and Alcohols



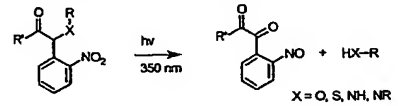

G. Linker for the formation of Sulfonamides and Alcohols



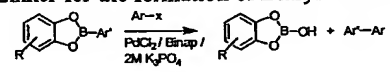

H. Linker for the formation of Ketones, Amines and Alcohols



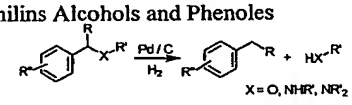

I. Linker for the formation of Ketones, Amines, Alcohols and Mercaptanes.



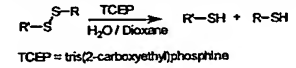

J. Linker for the formation of Biaryl and Bihetaryl



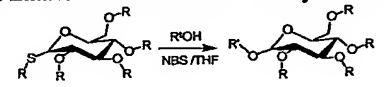

K. Linker for the formation of Benzyles, Amines, Anilins Alcohols and Phenoles



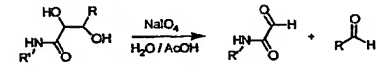

L. Linker for the formation of Mercaptanes




M. Linker for the formation of Glycosides




N. Linker for the formation of Aldehydes and Glyoxylamides




O. Linker for the formation of Aldehydes, Ketones And Aminoalcohols

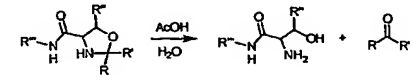


**Fig. 25. Templated synthesis by generating a new reactive group.**

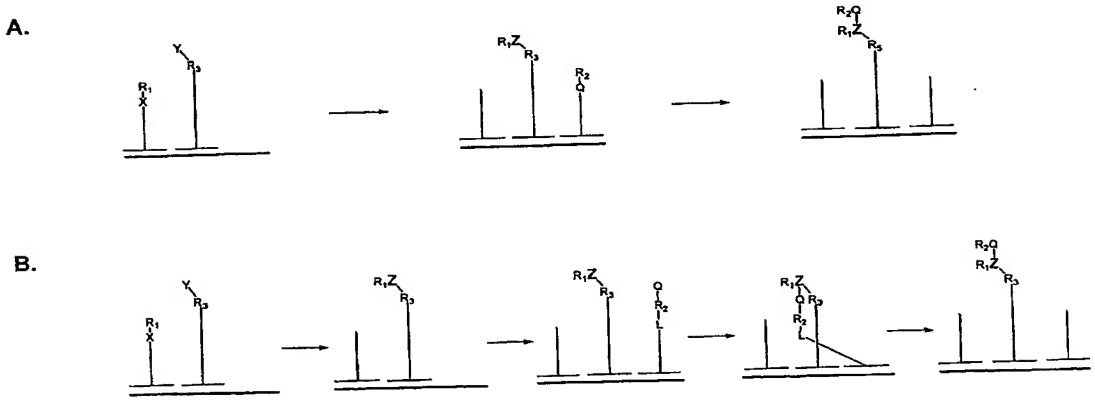


**Fig. 25, example 1. Generation of reactive groups in the first reaction round, followed by reaction of the generated reactive groups with introduced reactive groups.**

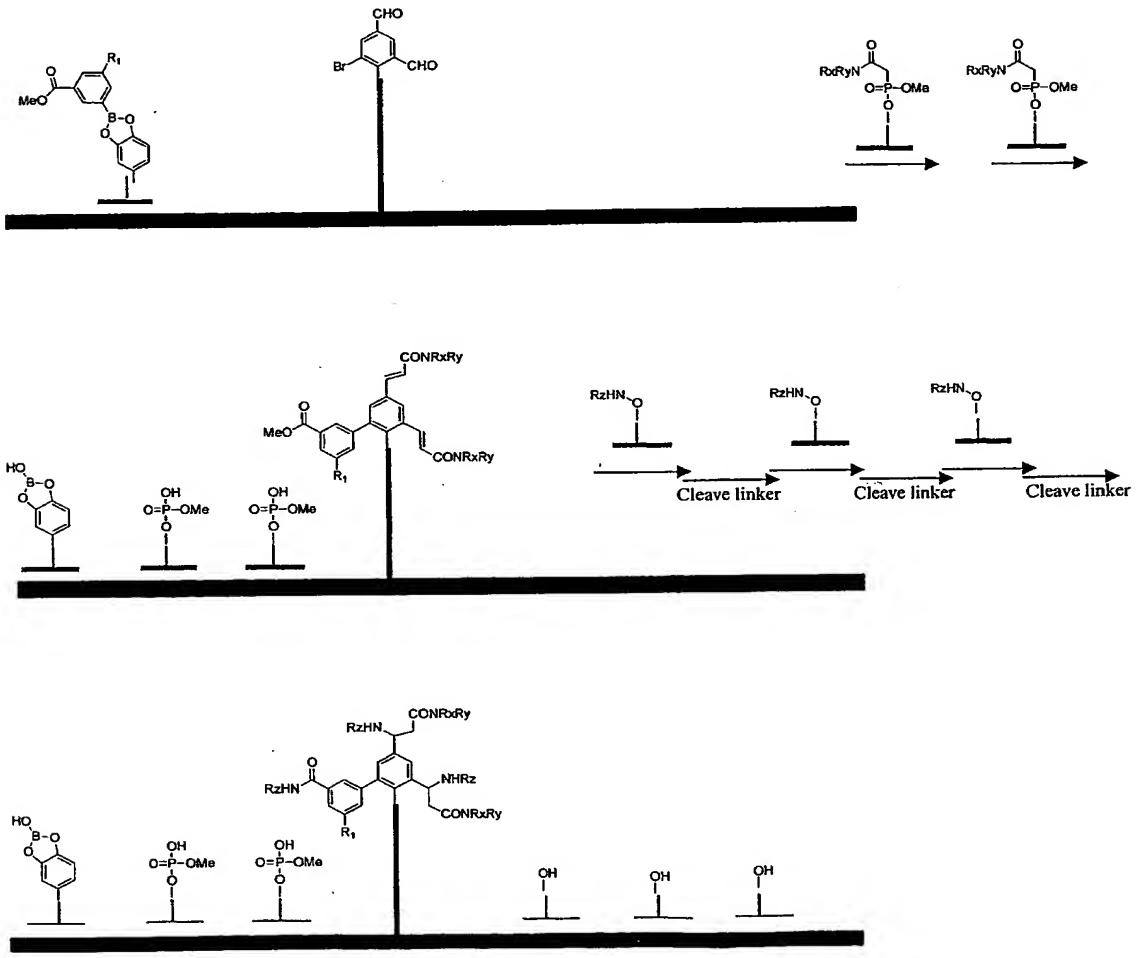
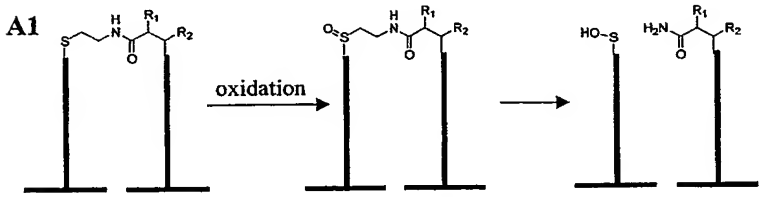


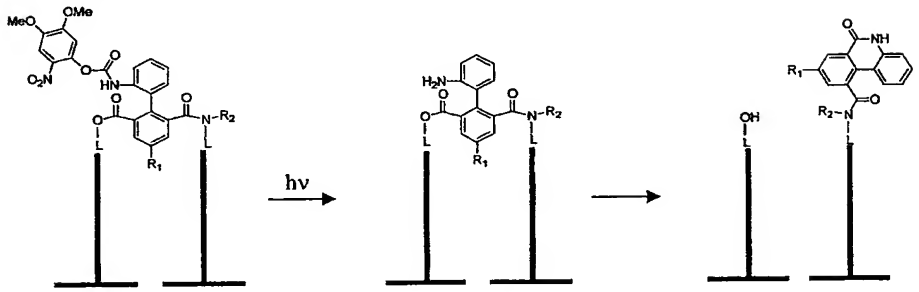
**Fig. 26. Post-templating modification of templated molecule**

A Rearrangement and cleavage in one step, eg:



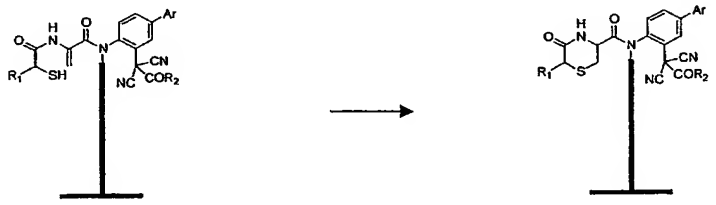

**A2**

Photo labile protecting group

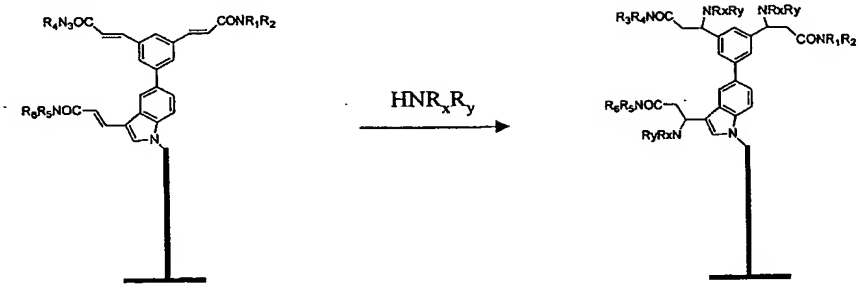



B Reaction of functional groups present in a templated molecule

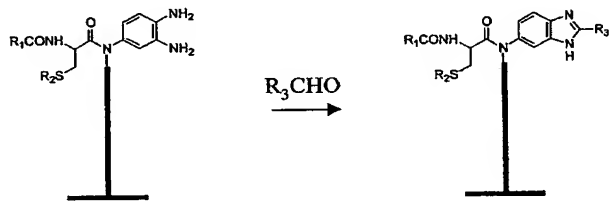
**B1** Intramolecular Michael addition:



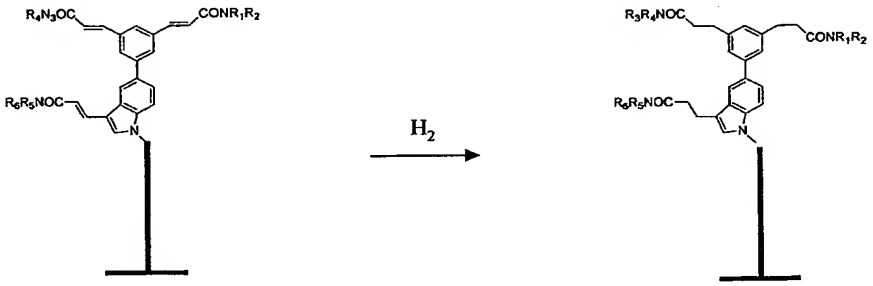
**B2** Intermolecular Michael addition:




**B3** Reaction of phenylenediamines and aldehydes to form benzimidazoles:




**B4** Reduction of multiple bonds:




**C** Post templating modification of linker to extend the spacing between the template and the templated molecule.

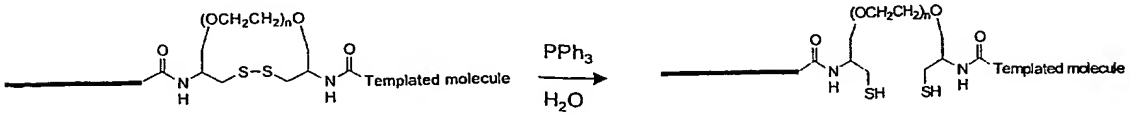
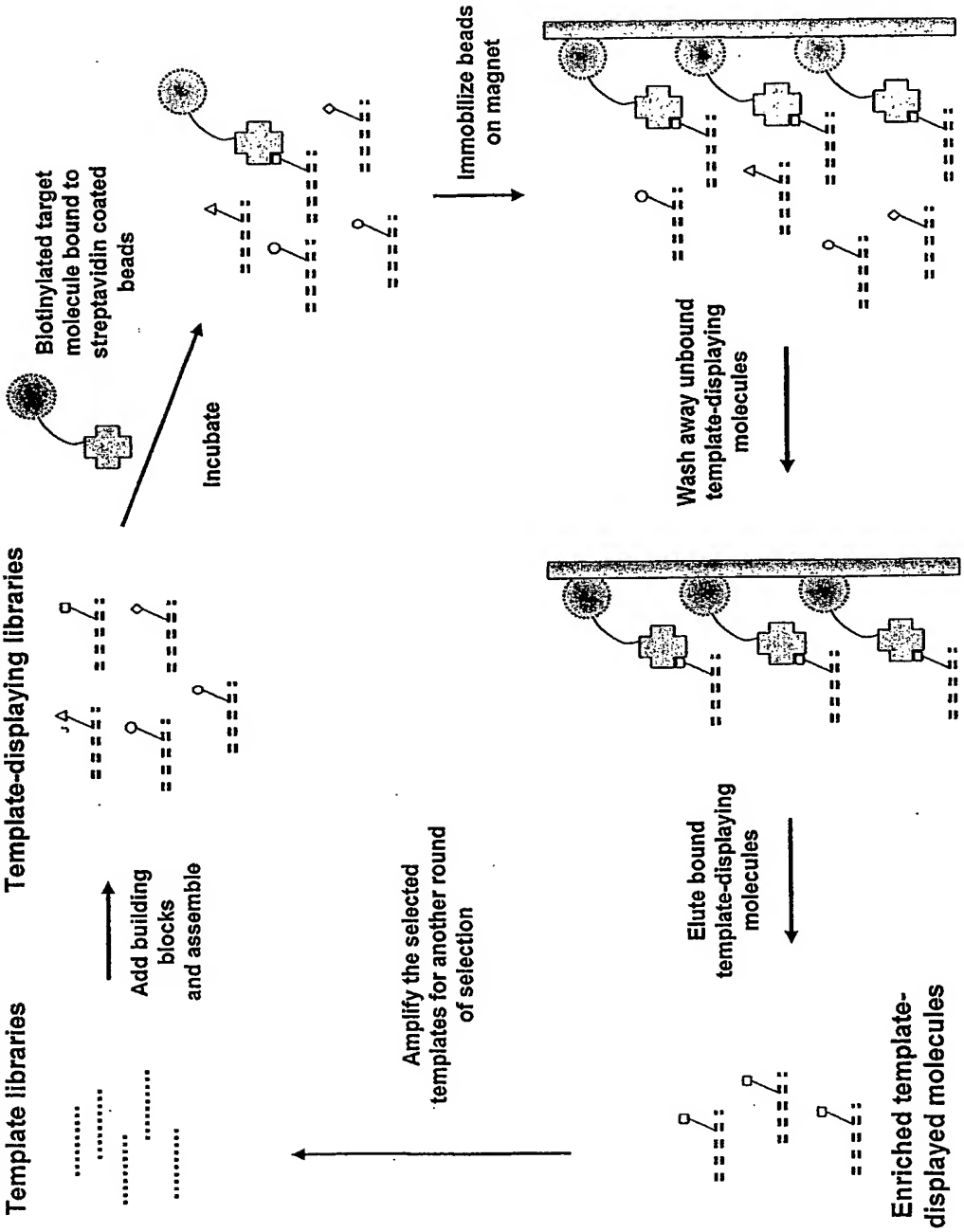


**Figure 27.**

**A typical panning protocol for selection of template-displaying molecules**



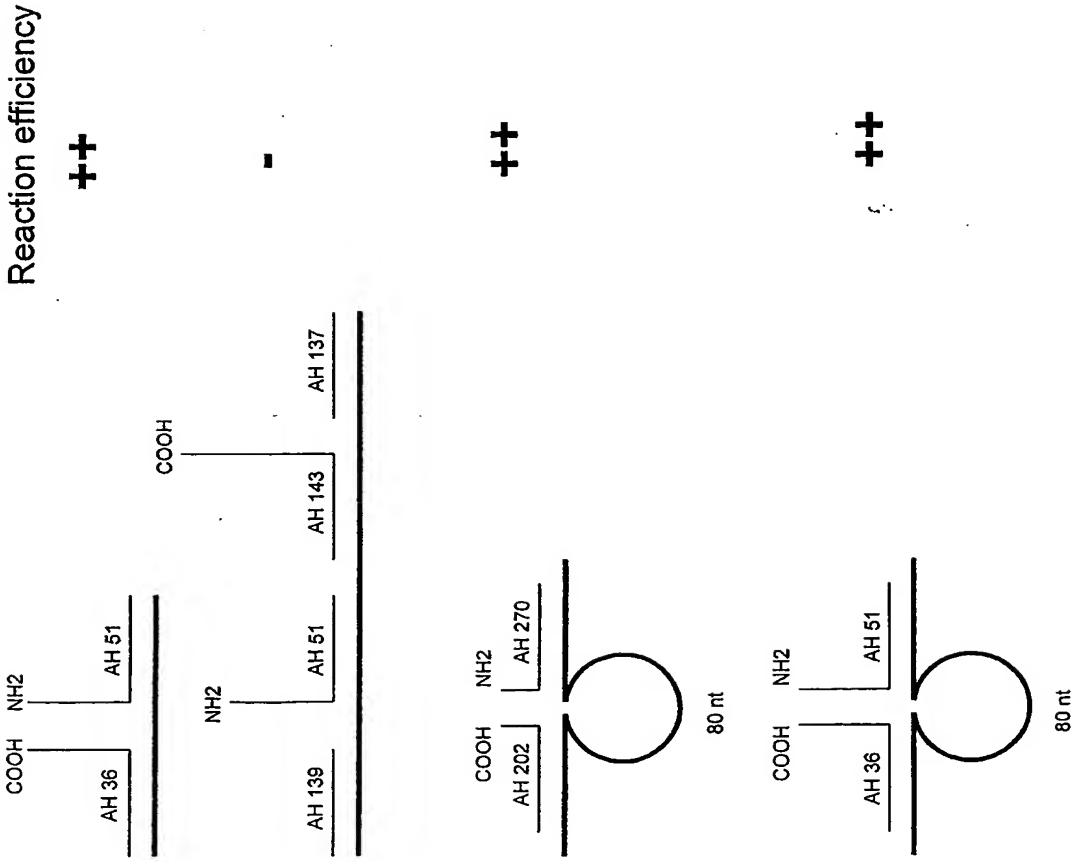
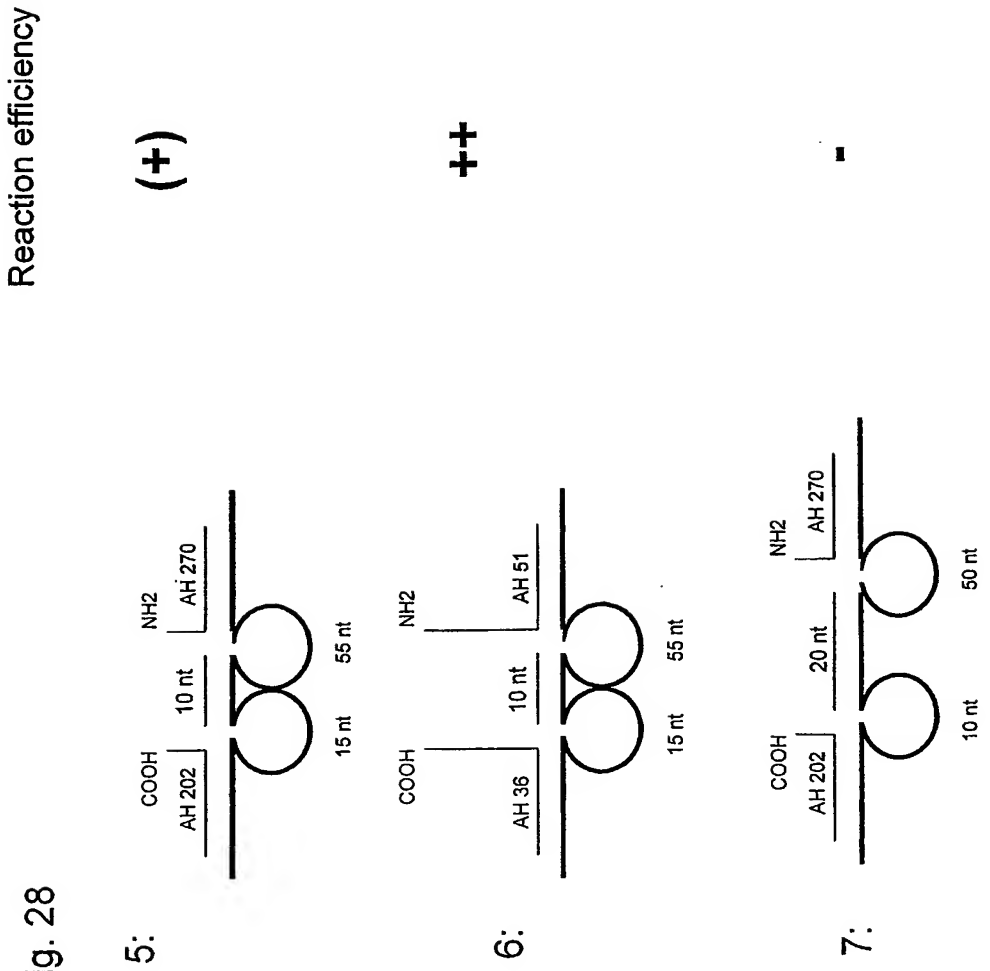

Reaction efficiency
++
-
++
++
1:
2:
3:
4:
COOH
NH2
AH 36
AH 51
AH 139
AH 143
AH 137
AH 202
AH 270
80 nt

Fig. 28

Fig. 28


Reaction efficiency
(+)
++
-
5:
COOH
AH 202
10 nt
NH2
AH 270
15 nt
55 nt
6:
COOH
AH 36
10 nt
NH2
AH 51
15 nt
55 nt
7:
COOH
AH 202
20 nt
NH2
AH 270
10 nt
50 nt

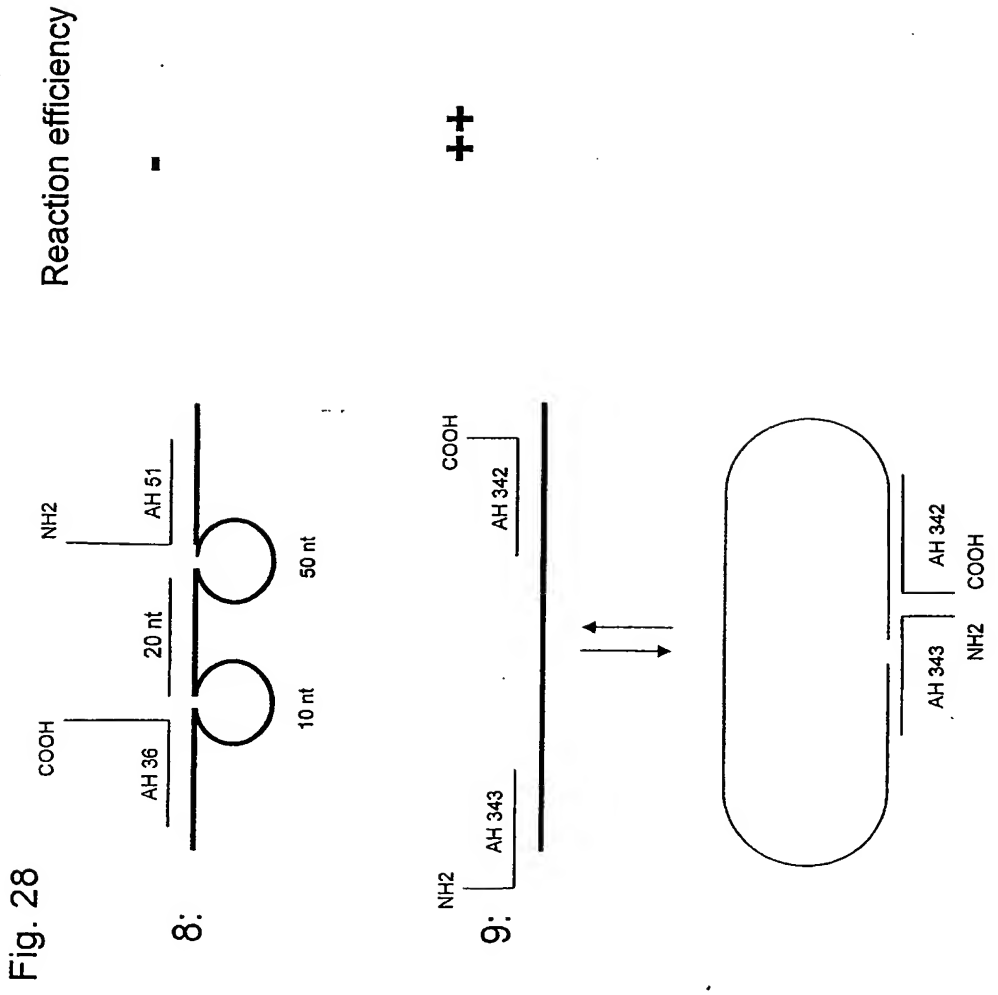
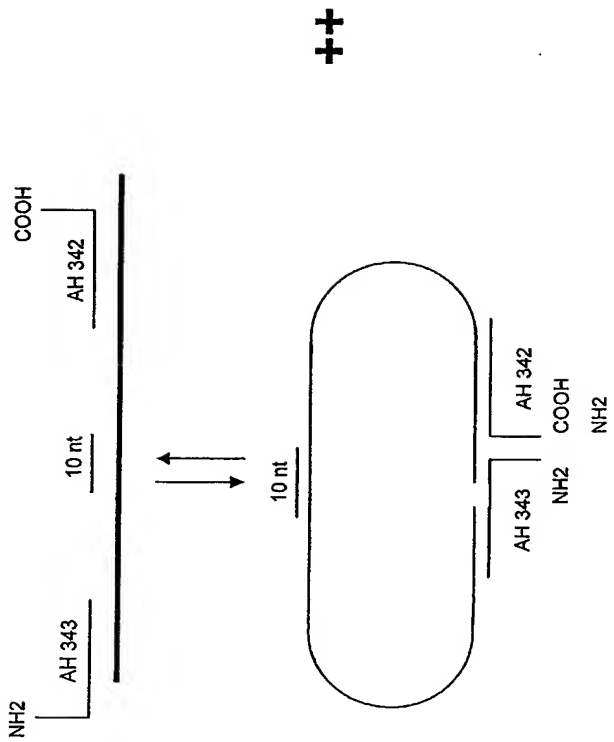

Reaction efficiency
-
++
8:
COOH
AH 36
20 nt
10 nt
NH2
AH 51
50 nt
9:
COOH
AH 342
NH2
AH 343
AH 343
NH2
AH 342
COOH

Fig. 28

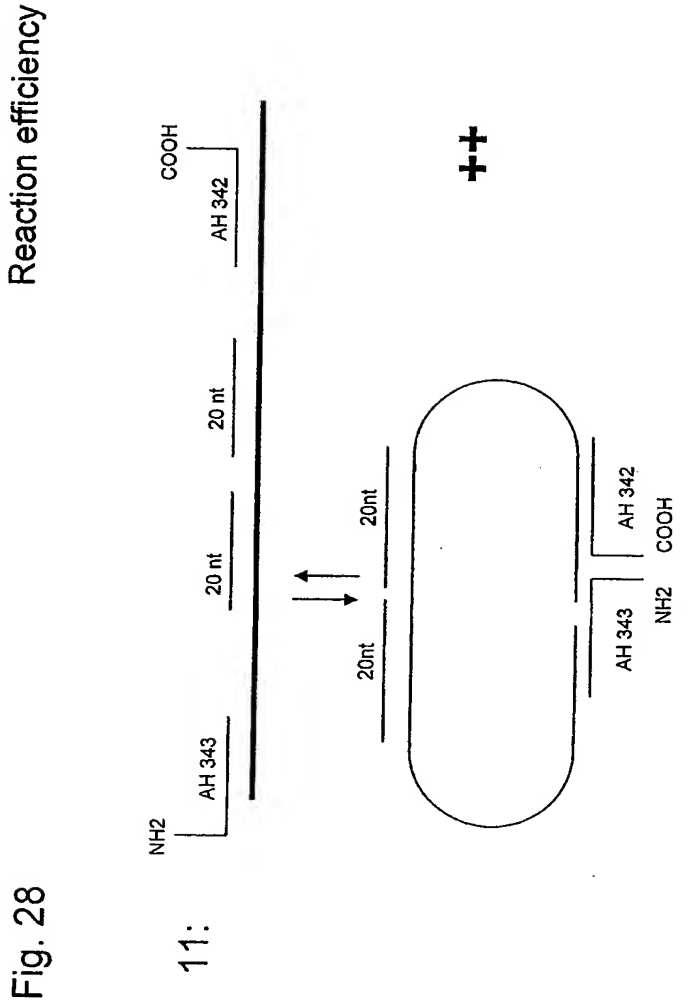
Reaction efficiency




10:

Fig. 28

Fig. 28


Reaction efficiency
11:
NH2
AH 343
20 nt
20 nt
COOH
AH 342
++
20nt
20nt
AH 343
NH2
AH 342
COOH

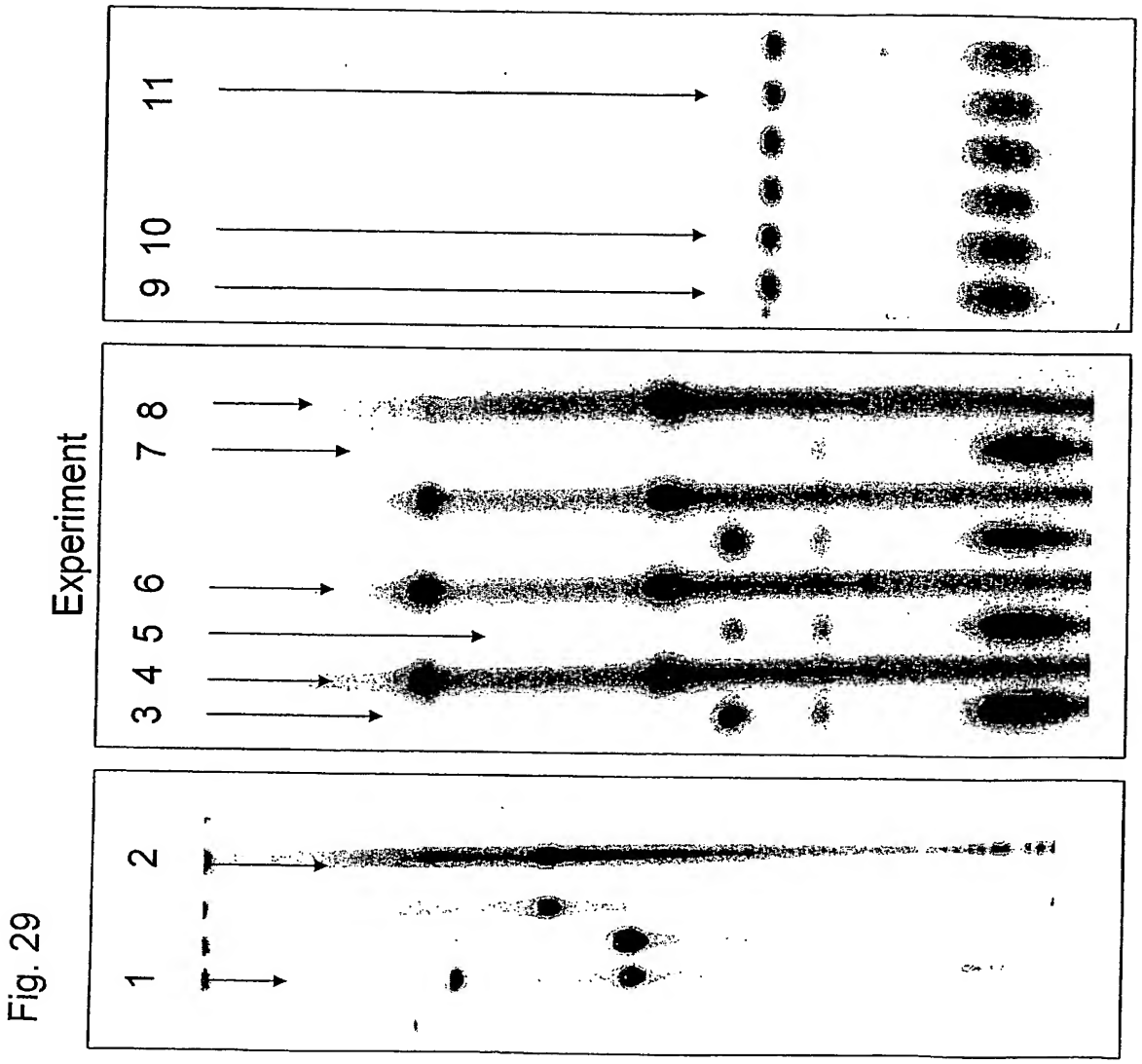
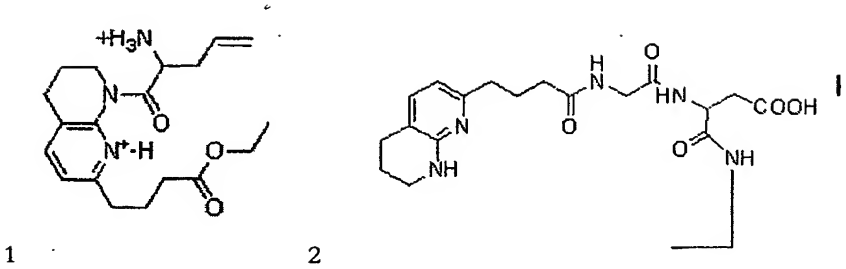



Fig. 29




1 2

**Figure 30.** Structure 1 shows the Feuston 3 functional entity, which is needed together with Gly and Asp to create Feuston 5 structure 2, a ligand that binds to the $\alpha_v\beta_3$ integrin receptor (as described in press; Feuston BP et al. J Med Chem. 2002 Dec 19;45(26):5640-8)

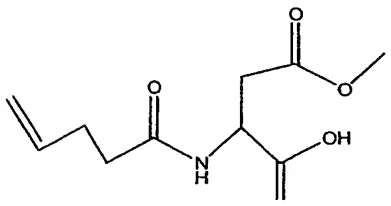



**Figure 31.** Structure of the pentenoyl protected aspartate functional entity used to load an amino modified scaffold oligo, to create the Feuston 5 ligand.

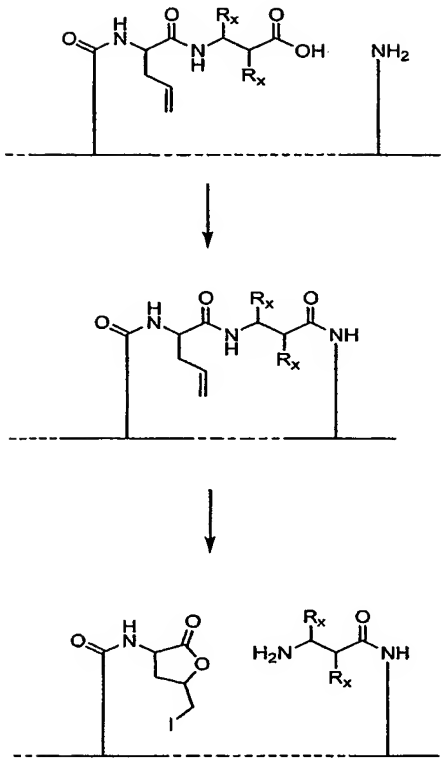


**Figure 32.** Allylglycine chemistry illustrated by structure showing cross-linked product as well as transferred product after cleavage by iodine.

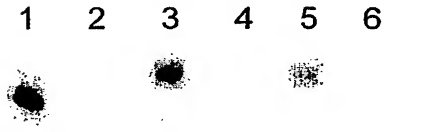



**Figure 33.** An autoradiography showing the three transfers of β-Ala to an amino modified scaffold oligo, this scaffold oligo being radioactively labeled. Lanes 1, 3 and 5 shows cross-linked product between scaffold amine and functional entity β-Ala AG carboxylic acid for transfers 1, 2 and 3. Lanes 2, 4 and 6 shows cleaved product, i.e. scaffold carrying the transferred functional entity.

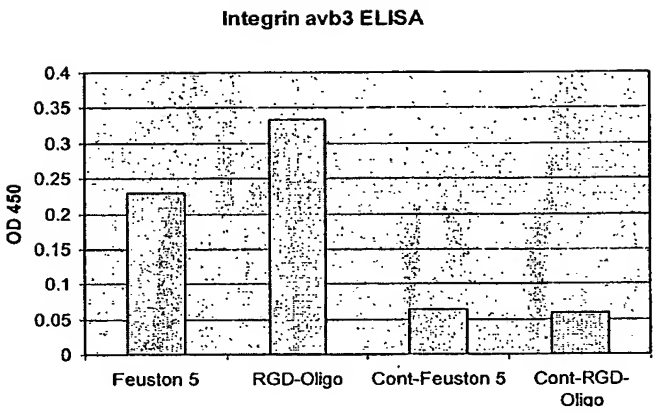



**Figure 34** Result from the ELISA done on the feuston 5 ligand generated by sequential transfers to a scaffold oligo (first column). The controls are the RGD peptide, which is an Integrin ligand (second column;) loaded on a 20 mer oligo, and uncoated wells (no Integrin immobilized; third and fourth columns).

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 February 2004 (12.02.2004)

PCT

(10) International Publication Number
WO 2004/013070 A3

(51) International Patent Classification[7]: C12N 15/10, C12P 21/00, C07H 21/00, C07B 61/00

(21) International Application Number: PCT/DK2003/000516

(22) International Filing Date: 30 July 2003 (30.07.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2002 01171 1 August 2002 (01.08.2002) DK
60/399,692 1 August 2002 (01.08.2002) US

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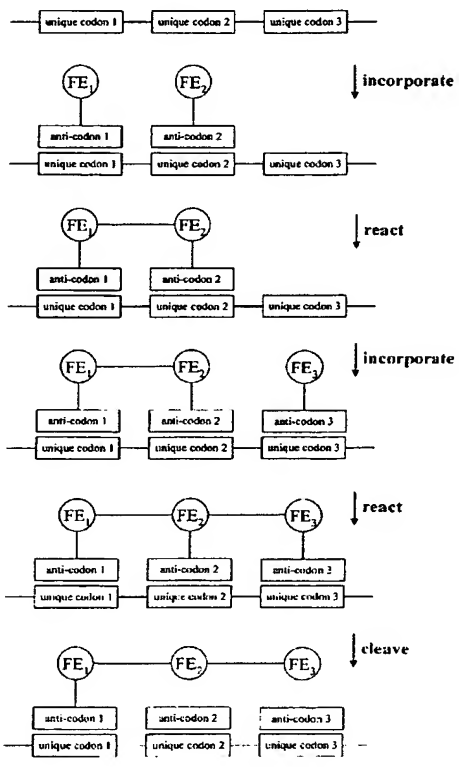
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

*[Continued on next page]*

(54) Title: MULTI-STEP SYNTHESIS OF TEMPLATED MOLECULES




(57) Abstract: Disclosed is a method for the manufacture of a library of complexes. The complexes comprise templated molecules attached to the template which directed the synthesis thereof. The templated molecules are produced in a step-by-step fashion which provides for a high local concentration of reactive groups involved in the formation of connections between the individual components of the template molecule.

WO 2004/013070 A3

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

**(88) Date of publication of the international search report:**
25 March 2004

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International Application No
PCT/DK 03/00516

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12P21/00 C07H21/00 C07B61/00

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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|---|---|---|
| P,X | WO 02 074929 A (KANAN MATTEW W;GARTNER ZEV J ; LIU DAVID R (US); HARVARD COLLEGE ()) 26 September 2002 (2002-09-26) figures 1-57 --- | 1-65 |
| P,X | WO 02 103008 A (GOUILAEV ALEX HAAHR ;NOERREGAARD-MADSEN MADS (DK); SLOEK FRANK ABI) 27 December 2002 (2002-12-27) figure 1 --- | 1-65 |
| X | WO 99 51546 A (HARVARD COLLEGE ;JACOBSEN ERIC N (US); SIGMAN MATTHEW S (US)) 14 October 1999 (1999-10-14) claims 1-54 --- | 1-65 |
| X | WO 00 23458 A (UNIV LELAND STANFORD JUNIOR) 27 April 2000 (2000-04-27) claims 1-14 --- | 1-65 |
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International Application No
PCT/DK 03/00516

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